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Tissue distribution and characteristics of canine non-conventional T cells

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To Johannes and Karl

Contents

Abbreviations

APC	antigen presenting cells
BALT	bronchus-associated lymphoid tissue
BCL6	B cell lymphoma 6 protein
β2m	β2-microglobulin
CD	cluster of differentiation
CTLA4	cytotoxic T lymphocyte antigen 4
CXCR5	C-X-C motif chemokine receptor 5
dn	double-negative
dp	double-positive
e.g.	*exempli gratia* / for example
EOMES	Eomesodermin
FoxP3	forkhead box p3
GALT	gut-associated lymphoid tissue
IBD	inflammatory bowel disease
i.e.	*id est* / that is to say
IEL	intraepithelial lymphocytes
IFN-γ	interferon γ
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
MAIT cell	mucosa-associated invariant T cell
MALT	mucosa-associated lymphoid tissue
MHC	major histocompatibility complex
MHCI	major histocompatibility complex class I
MHCII	major histocompatibility complex class II
MR1	major histocompatibility complex class I-related gene protein
NK cell	natural killer cell
NKG2D	natural killer group 2, member D
NKT cell	natural killer T cell

PAMP	pathogen-associated molecular patterns
PMA	phorbol-myristate-acetate
PP	Peyer’s patches
PRR	pattern recognition receptors
RACE	rapid amplification of cDNA ends
RAE1	retinoic acid early transcript 1
resp.	respectively
RORγT	retinoic acid receptor-related orphan receptor γT
SLE	systemic lupus erythematosus
sp	single-positive
T-bet	T-box transcription factor TBX21
TCM	central memory T cell
TCR	T cell receptor
TCRαβ	T cell receptor αβ
TCRγδ	T cell receptor γδ
TEM	effector memory T cell
Tfh	follicular T helper cell
TGF-β	transforming growth factor β
Th	T helper cell
Th1	Type 1 T helper cell
Th2	Type 2 T helper cell
Th9	Type 9 T helper cell
Th17	Type 17 T helper cell
TNF-α	tumor necrosis factor α
Treg	regulatory T cell
vs.	versus

1 Introduction

Adequate healthcare for animals gains more and more in importance. This includes on the one hand professional specialization in veterinary medicine, and on the other hand an in-depth understanding of the physiological and pathophysiological processes in different species. This dissertation is dedicated to the dog, our beloved companion as pet, experimental or working dog whose immune system is still not fully understood.

1.1 The innate and adaptive immune system of mammals

In general, the immune system of mammals can be divided into an innate and an adaptive branch. Both include cellular and humoral components. Cells of the innate immune system, like phagocytes (e.g. macrophages, dendritic cells, neutrophil granulocytes) or natural killer (NK) cells constitute the first line of defense against invading pathogens. Usually, they are activated by common structural characteristics of pathogens, so-called pathogen-associated molecular patterns (PAMPs) (e.g. lipopolysaccharide (LPS) found on all gram-negative bacteria, see WANG and QUINN 2010) binding to pattern recognition receptors (PRR) (JANEWAY and MEDZHITOV 2002).

In a second step, cells of the adaptive immune system, i.e. T and B cells (lymphocytes) are activated. Their response is more target-oriented. They are capable of recognizing unique structural characteristics of one special pathogen (e.g. the gram-negative bacterium *Pseudomonas aeruginosa*). These specific structures are called antigens. Another feature of T and B cells is the induction of immunological memory. This means that once the specific B or T cell has encountered its specific antigen, the immune response on the second encounter is much faster and more efficient (BONILLA and OETTGEN 2010, NETEA et al. 2019). B cells are known as effective antibody producers and can present antigen to T cells. Further functions and subtypes of B cells can be found elsewhere (SEIFERT and KÜPPERS 2016).

1.2 Conventional αβ T cells

Each T cell has a T cell receptor enabling it to bind to its specific antigen. The T cell receptor of conventional T cells is T cell receptor αβ (TCRαβ), a heterodimer consisting of variable α and β polypeptide chains. TCRαβ forms a complex with cluster of differentiation (CD) 3, which is essential for the intracellular signaling cascade (CLEVERS et al. 1988). As CD3 can be found on all T cells independent of the T cell receptor subtype, it is used as a global T cell marker to detect T cells by flow cytometry (MOUSSET et al. 2019) or immunohistology (CHETTY and GATTER 1994).

TCRαβ binds to antigen, which is either presented by professional antigen presenting cells (APC), e.g. macrophages, dendritic cells, or B cells via major histocompatibility complex class II (MHCII) or by any body cell via MHCI. It depends on the αβ T cell subtype whether it binds to antigen presented

via MHCI or II: The two main subtypes are CD8αβ$^+$ cytotoxic T cells and CD4$^+$ T helper resp. regulatory T cells. The nomenclature originates from the corresponding T cell co-receptor found on their surface (GERMAIN 2002, WIECZOREK et al. 2017). The binding of the co-receptor to conserved regions of MHC is important for optimal activation of the αβ T cell (KÖNIG 2002).

1.2.1 Cytotoxic CD8αβ$^+$ T cells

Conventional cytotoxic T cells are characterized by expression of CD8αβ, a heterodimeric molecule consisting of two polypeptide chains α and β (ZAMOYSKA 1994) (Figure 1A). Even though a CD8αα homodimer exists, there is evidence that it does not work as a TCRαβ co-stimulatory molecule (CHEROUTRE and LAMBOLEZ 2008, GANGADHARAN and CHEROUTRE 2004, MCNICOL et al. 2007). CD8αβ$^+$ cytotoxic T cells control the body cells for possibly invading intracellular pathogens (e.g. viruses), which are presented to them via MHCI. Cytotoxic T cells can also be able to detect genetic changes in body cells, such as tumor cells (ANDERSEN et al. 2006). Once the target cell is identified, the cytotoxic T cell secretes perforin and granzymes into the intercellular space. Perforin leads to a permeability of the target cell's surface membrane, through which the granzymes can enter the cell interior and initiate apoptosis (TRAPANI and SMYTH 2002). Alternatively, apoptosis can be induced by interaction between the Fas receptor on the target cell and the Fas ligand on the cytotoxic T cell (WARING and MÜLLBACHER 1999). An activated cytotoxic T cell releases the cytokines interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) to enhance cytotoxicity (ANDERSEN et al. 2006). The key transcription factors for cytotoxic T cells to gain effector functions are T-box transcription factor TBX21 (T-bet) and Eomesodermin (EOMES) (LAZAREVIC et al. 2013).

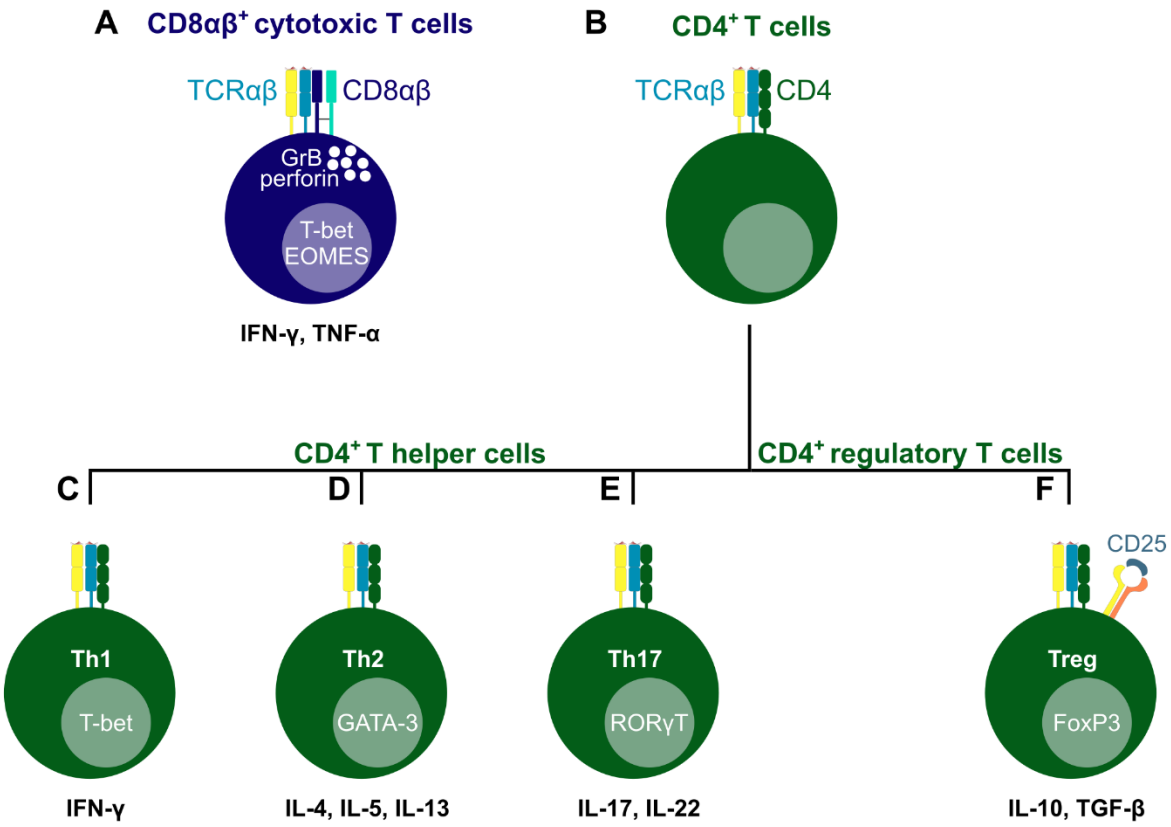

Figure 1: Subsets of conventional αβ T cells. Conventional αβ T cells consist of CD8αβ$^+$ cytotoxic (A) and CD4$^+$ T cells (B). The latter comprise T helper (Th, C - E) and regulatory (Treg, F) T cells. Depicted are the secreted proteins granzyme B and perforin resp. T helper subtypes (written in white within cytosol), master transcription factors (written in white within nuclei) and main cytokines (written in black outside the cell). TCR: T cell receptor; CD: Cluster of Differentiation; GrB: granzyme B; T-bet: T-box transcription factor TBX21; EOMES: Eomesodermin; IFN-γ: interferon γ; IL: interleukin; RORγT: retinoic acid receptor-related orphan receptor-γT; FoxP3: forkhead box P3; TGF-β: transforming growth factor β

1.2.2 CD4$^+$ T cells

A common feature of all CD4$^+$ αβ T cells is the binding of their T cell receptor to antigen presented via MHCII on professional APC (WIECZOREK et al. 2017). Once activated, they develop either into T helper (Th) or into regulatory T cells (Treg). The various Th cell subtypes as well as Treg can be differentiated according to their specific cytokine pattern and lineage-specific master transcription factors (ZHU 2018) (Figure 1B – F).

1.2.2.1 CD4$^+$ T helper cells

Type 1 T helper cells (Th1) play a crucial role during infections with intracellular pathogens. Typical examples are infections with *Mycobacterium tuberculosis* (JASENOSKY et al. 2015) or *Leishmania infantum* (TOEPP and PETERSEN 2020). The development of Th1 is regulated by the transcription

factor T-bet (Figure 1C). T-bet activates the characteristic effector cytokine IFN-γ, which leads for instance to classical activation of macrophages and increased MHCI and MHCII expression (SZABO et al. 2000, SZABO et al. 2002, SZABO et al. 2003).

By production of the cytokine interleukin 4 (IL-4), Type 2 T helper cells (Th2) contribute to antibody class switch of B cells from immunoglobulin (Ig) M to IgG1 or IgE (JUNTTILA 2018, KOPF et al. 1993). Furthermore, release of IL-5 and IL-13 leads to recruitment of eosinophils resp. increased mucus production and bronchoconstriction (FINKELMAN et al. 2010). Therefore, Th2 are essential for humoral immune responses, during helminth infections and pathogenesis of allergic diseases (NAKAYAMA et al. 2017). ZHANG et al. 1997 and ZHENG and FLAVELL 1997 found that GATA-3 is the key transcription factor for the development of Th2 (Figure 1D).

During the last 20 years, it has become apparent that there are further subtypes besides classical Th1 and Th2. A T helper lineage characterized by the production of IL-17 was discovered and called Th17 cells (LANGRISH et al. 2005, MCKENZIE et al. 2006, WEAVER et al. 2006). Shortly afterwards retinoic acid receptor-related orphan receptor γT (RORγT) was identified as crucial transcription factor for Th17 specific cytokine production (IVANOV et al. 2006). Nowadays, IL-17 and IL-22 are regarded as main cytokines of Th17 (Figure 1E). IL-17 is a pro-inflammatory cytokine providing protection at epithelial barriers, e.g. by attracting neutrophil granulocytes. IL-22, on the other hand, is mainly a homeostatic cytokine characterized for instance by promotion of epithelial repair. Hence, Th17 are involved in the pathogenesis of a variety of autoimmune diseases, e.g. psoriasis (EYERICH et al. 2017).

In addition to Th17, several T helper subtypes have been identified: Amongst others, follicular T helper cells (Tfh) specialized on B cell help (CROTTY 2011), or Type 9 T helper cells (Th9) (KAPLAN et al. 2015) should be mentioned here.

1.2.2.2 $CD4^+$ regulatory T cells

In contrast to pro-inflammatory cytotoxic $CD8\alpha\beta^+$ cytotoxic and $CD4^+$ T helper cells, $CD4^+$ regulatory T cells (Treg) are responsible for avoiding an exuberant immune response and thus prevent autoimmunity (CORTHAY 2009). In the nineties, a suppressive $CD4^+$ T cell subtype which produces the anti-inflammatory cytokines IL-10 and transforming growth factor β (TGF-β) was discovered (CHEN et al. 1994, GROUX et al. 1997). Furthermore, Treg express co-inhibitory molecules like cytotoxic T lymphocyte antigen 4 (CTLA4) to downregulate professional APC (ROWSHANRAVAN et al. 2018). Deficiency in Treg causes the development of severe autoimmune diseases, whereas a high number of Treg in cancer environment can lead to the suppression of effector T cells resulting in tumor growth (LU et al. 2017).

SAKAGUCHI et al. 1995 could show that the maintenance of Treg is dependent on IL-2, an important T cell growth factor, and that Treg constitutively express CD25, the IL-2 receptor α-chain. Hence, CD25 is a useful marker to identify Treg (SAKAGUCHI et al. 1995, SAKAGUCHI et al. 2007). Of note, IL-2 is an important cytokine for the generation of effector and memory T cells and activated $CD4^+$ T helper and $CD8\alpha\beta^+$ cytotoxic T cells upregulate CD25 as well (ABBAS et al. 2018). Since the identification of the transcription factor forkhead box P3 (FoxP3) as master transcription factor of Treg, they can be described as $CD4^+CD25^+FoxP3^+$ Treg (FONTENOT et al. 2003, HORI 2003, KHATTRI et al. 2003) (Figure 1F).

1.3 Non-conventional T cells

The term 'unconventional T cells' is used by GODFREY et al. 2015 and PELLICCI et al. 2020 to describe T cells in humans and mice that do not recognize peptide antigen via the classical MHC-TCRαβ pathway. This group of T cells mainly comprises natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells and γδ T cells (GODFREY et al. 2015, PELLICCI et al. 2020). NKT cells are characterized by expression of a semi-invariant TCRαβ which binds to lipid antigens presented by CD1d (TANIGUCHI et al. 2015). In dogs, expression of CD1d (and with it the existence of functional NKT cells *in vivo*) might be divergent due to structural differences of the canine CD1d gene in comparison to humans (VAN LOORINGH BEECK et al. 2013). MAIT cells are specialized for the recognition of microbial-derived vitamin B metabolites presented by major histocompatibility complex class I-related gene protein (MR1) (GODFREY et al. 2019, PELLICCI et al. 2020).

In order to include T cells whose pathway of antigen recognition is not yet fully understood, 'non-conventional T cells' are defined here as T cells that differ from conventional αβ T cells either in co-receptor or TCR expression.

1.3.1 $CD4^+CD8\alpha^+$ double-positive T cells

T cells that express CD4 as well as CD8 (CD8αα or CD8αβ, hereafter referred to as $CD8\alpha^+$) are known as an immature stage during T cell development in the thymus (KOCH and RADTKE 2011). However, in several species (e.g. monkeys, humans, chicken, mice, pigs, horses and rats) mature $CD4^+CD8\alpha^+$ double-positive (dp) T cells could be isolated from peripheral blood or tissues (AKARI et al. 1997, BLUE et al. 1986, LUHTALA et al. 1997, MOSLEY et al. 1990, RANERA et al. 2016, SAALMÜLLER et al. 1987, TAKIMOTO et al. 1992). They differ from thymocytes in the lack of thymic markers, and in their expression of activation and/or memory markers (OVERGAARD et al. 2015, ZUCKERMANN 1999). As described above, T cells become activated upon antigenic stimulation and differentiate into effector cells. After successful elimination of the pathogen, the majority of effector T cells dies. However, a pool of memory T cells remains either in lymphoid organs (i.e. central

memory T cells, TCM) or migrates to the tissues (effector memory T cells, TEM). In a second encounter of pathogen and memory T cell, the immune response is accordingly faster (JAMESON and MASOPUST 2018). In swine, activation and memory formation of $CD4^+$ Th is associated with upregulation of CD8αα. This results in a $CD4^+CD8αα^{dim}$ dp phenotype that makes up to 60% of all T cells in an age-dependent manner (PESCOVITZ et al. 1994, SAALMÜLLER et al. 1987). Porcine $CD4^+CD8αα^{dim}$ dp memory T cells are either $CD27^+$ with high proliferative capacity and IL-2 production (referred to as TCM), or $CD27^-$ rather producing IFN-γ and TNF-α with less proliferative potential (referred to as TEM) (GERNER et al. 2015). SUMMERFIELD et al. 1996 showed a strong proliferation of $CD4^+CD8αα^{dim}$ dp T cells from pigs immunized with pseudorabies virus upon viral recall antigenic stimulation. Thus, $CD4^+CD8αα^{dim}$ dp T cells of pigs are a widely accepted T cell subpopulation involved in the immune response to various pathogens (reviewed by GERNER et al. 2015). In the human species, $CD4^+CD8α^+$ dp T cells seem to play a role in several diseases, especially autoimmune disorders, infections, or cancer (NISHIDA et al. 2020, OVERGAARD et al. 2015). Following subcutaneous vaccination, $CD4^+CD8α^+$ dp T cells accumulate in the skin of mice suggesting potent functions during immune response (OVERGAARD et al. 2017). To exclude that $CD4^+CD8α^+$ dp T cells could primarily be aggregates of single-positive (sp) cells (KELLY et al. 1988), OVERGAARD et al. 2017 showed via Amnis ImageStream - a method that combines flow cytometry with microscopy - that they definitely exist in mice. For canine peripheral blood $CD4^+CD8α^+$ dp T cells, this was confirmed by our group (unpublished data).

First reports about a $CD4^+CD8α^+$ dp T cell population in dogs came up during analyses of canine umbilical cord blood (OTANI et al. 2008), and in context with canine leishmaniasis (ALEXANDRE-PIRES et al. 2010). At the same time, SCHÜTZE et al. 2009 found that canine $CD4^+CD8α^+$ dp T cells proliferate upon restimulation with *Parapoxvirus ovis*. This interesting observation led to a comprehensive characterization of canine peripheral blood $CD4^+CD8α^+$ dp T cells revealing their mature extrathymic (i.e. lack of the thymic marker CD1a) $TCRαβ^+$ phenotype (BISMARCK et al. 2012). In addition, most of these cells constitutively express markers of activation (CD25, MHC-II) and exhibit an effector memory phenotype with high potential for IFN-γ production (BISMARCK et al. 2012, ROTHE et al. 2017). Canine $CD4^+CD8α^+$ dp T cells of peripheral blood are not a homogeneous population, but can be divided into three subpopulations: $CD4^{bright}CD8α^{dim}$, $CD4^{bright}CD8α^{bright}$, and $CD4^{dim}CD8α^{bright}$ (BISMARCK et al. 2012). Particularly noteworthy is the $CD4^{bright}CD8α^{bright}$ subpopulation, as it is the population with the highest activation status and most effector memory cells (ROTHE et al. 2017). Interestingly, the CD8α molecule of this subpopulation is not CD8αβ, but CD8αα (BISMARCK et al. 2012), raising questions about the function and origin of this subpopulation. An elegant study was performed by BISMARCK et al. 2014 showing that canine peripheral blood $CD4^+CD8α^+$ dp T cells mostly develop from $CD4^+$ single-positive (sp) T cells by upregulation of CD8α. Nevertheless, to a limited extent canine $CD8α^+$ sp T cells are also capable of developing into $CD4^+CD8α^+$ dp T cells (BISMARCK et al. 2014). In summary, these data indicate that canine

$CD4^+CD8\alpha^+$ dp T cells could play an important role in the immune response and pathologies of dogs. However, a limitation of these studies is their focus on peripheral blood. In that way, tissue-resident T cells that do not (or only shortly) appear in the circulation, but which are crucial for local protection and pathogenesis of multiple diseases in humans, are missed out (CLARK 2015, MASOPUST and SOERENS 2019). Therefore, an analysis of $CD4^+CD8\alpha^+$ dp T cells in canine lymphoid and non-lymphoid tissues was still pending.

1.3.2 γδ T cells

Apart from TCRαβ, T cells can express another type of T cell receptor consisting of a γ and a δ chain, termed TCRγδ (PRINZ et al. 2013). In humans and mice, they only comprise a small fraction of all circulating T cells, yet they can be found more abundantly at epithelial surfaces, such as the respiratory, genital, or digestive tract (CHIEN et al. 2014). Most human γδ T cells are $CD4^-CD8\alpha^-$ double-negative (dn) (PISTOIA et al. 2018). This is also the case with ruminants and pigs, and interestingly, numerous γδ T cells can be found in peripheral blood of these species (GUERRA-MAUPOME et al. 2019, SAALMÜLLER et al. 1990). Research in mice and humans revealed that γδ T cells take an intermediate position between the innate and adaptive immune system. Antigen recognition is not restricted to peptides presented via MHCI or II. They rather recognize different stress-induced stimuli (e.g. microbial and endogenous phosphoantigens or retinoic acid early transcript 1, RAE1) not only via TCRγδ, but also via the natural killer receptor NKG2D or PRR such as Toll-like receptors and dectin 1. This enables γδ T cells to rapid activation and on site reaction (BONNEVILLE et al. 2010, VANTOUROUT and HAYDAY 2013). Regarding effector functions, γδ T cells are able to exert cytotoxicity by secretion of perforin and granzymes resp. induction of the Fas-pathway (CARDING and EGAN 2002, DIELI et al. 2001, QIN et al. 2009). In mice, humans, and ruminants γδ T cells are considered as main source of IL-17 besides Th17 (AKITSU and IWAKURA 2018, GUERRA-MAUPOME et al. 2019, SILVA-SANTOS et al. 2015). Furthermore, they can secrete the effector cytokines of Th1 (TNF-α and IFN-γ), and Th2 (IL-4, IL-5, IL-13) as well as Treg (TGF-β, IL-10) with corresponding effects (BONNEVILLE et al. 2010). As part of tissue-resident intraepithelial lymphocytes (IEL), γδ T cells contribute to tissue homeostasis and repair (NIELSEN et al. 2017).

With the development of a monoclonal antibody specific for canine TCRγδ, studies on the distribution of canine γδ T cells in peripheral blood and tissues became possible. Immunohistological and flow cytometric examination of the canine intestine revealed a large portion of γδ T cells in the intestinal epithelium with an age-dependent $CD4^-CD8\alpha^-$ dn (neonates) or $CD8\alpha\alpha^+$ single-positive (sp) phenotype (GERMAN et al. 1999, LUCKSCHANDER et al. 2009). Similar to humans and mice, canine γδ T cells only comprise a minor portion in peripheral blood and lymphatic organs (GIBSON et al. 2004, FALDYNA et al. 2005, LUCKSCHANDER et al. 2009, RÜTGEN et al. 2015) which even decreases

in dogs with inflammatory bowel disease (IBD) (GALLER et al. 2017). Further analyses concerning phenotype and function of canine γδ T cells were still missing.

1.3.3 $CD4^-CD8\alpha^-$ double-negative αβ T cells

αβ T cells that neither express CD4 nor CD8α represent a small subpopulation of approximately 1 – 3% of all $CD3^+$ T cells in peripheral blood of humans and mice (FISCHER et al. 2005, ZHANG et al. 2000). In higher numbers they are found in the murine and human kidney (ASCON et al. 2008, MARTINA et al. 2016) or the murine female genital tract (JOHANSSON and LYCKE 2003). Human $TCR\alpha\beta^+CD4^-CD8\alpha^-$ dn T cells comprise naïve and antigen-experienced effector cells mainly producing IFN-γ as well as IL-4, IL-5 and IL-10 (FISCHER et al. 2005, VOELKL et al. 2011). The most intriguing function of these cells in humans as well as mice is their ability to control immune responses both *in vitro* and *in vivo*. Therefore, they are also termed dn regulatory T cells (dn Treg) (FISCHER et al. 2005, TIAN et al. 2019, VOELKL et al. 2011, ZHANG et al. 2001), yet they lack expression of FoxP3 and barely express CD25 (FISCHER et al. 2005, HILLHOUSE et al. 2010, PRIATEL et al. 2001, VOELKL et al. 2011, ZHANG et al. 2007). In this context dn Treg are able to prevent allograft rejection (CHEN et al. 2003, ZHANG et al. 2000), graft-versus-host disease (HE et al. 2007, MCIVER et al. 2008, YOUNG et al. 2002, YOUNG et al. 2003), or auto-immune diabetes (DUGAS et al. 2010, DUNCAN et al. 2010, FORD et al. 2007, ZHANG et al. 2011). On the other hand, involvement of $TCR\alpha\beta^+CD4^-CD8\alpha^-$ dn T cells in the pathogenesis of autoimmune diseases by production of pro-inflammatory cytokines like IL-17A or IFN-γ indicates their potentially detrimental function (reviewed by BRANDT and HEDRICH 2018). Human $TCR\alpha\beta^+CD4^-CD8\alpha^-$ dn T cells were implicated in systemic lupus erythematosus (SLE) (CRISPÍN et al. 2008, DEAN et al. 2002, LAI et al. 2013), psoriasis (BRANDT et al. 2017, UEYAMA et al. 2017) or myasthenia gravis (REINHARDT and MELMS 2000), which are also diseases occurring in dogs.

Canine $TCR\alpha\beta^+CD4^-CD8\alpha^-$ dn T cells have often been neglected in flow cytometric analyses. So, only single reports on this T cell subpopulation exist for dogs (ALEXANDRE-PIRES et al. 2010, LUCKSCHANDER et al. 2009, MAINA et al. 2019) and no further characterization had been performed yet.

1.4 Aims of this study

The aim of this study was to provide an overview on the occurrence and phenotype of non-conventional T cells in canine peripheral blood and tissues. This is the basis for further studies regarding *in vivo* regulatory and/or effector functions of these cells and their role in diseases of dogs.

The first part of this work extends earlier studies of $CD4^+CD8\alpha^+$ dp T cells in peripheral blood (BISMARCK et al. 2012, BISMARCK et al. 2014, ROTHE et al. 2017) by characterization of $CD4^+CD8\alpha^+$

dp T cells in lymphatic (lymph nodes, spleen, Peyer's patches, PP) and non-lymphatic organs (lung and epithelium of the small intestine) of healthy Beagle dogs. Of interest were tissue-specific expression patterns of TCRαβ vs. TCRγδ, CD8αβ vs. CD8αα, the activation marker CD25, the Treg transcription factor FoxP3 and also the cytotoxic molecule granzyme B.

The second part of this work is a first characterization of canine TCRαβ^{+}CD4^{-}CD8α^{-} dn and γδ T cells in peripheral blood and tissues mentioned above. We were interested in frequencies and tissue distribution. Moreover, for defining their functional potential expression of key transcription factors (e.g. T-bet, GATA-3, FoxP3), surface markers (e.g. CD25, CD5), and cytokines (IFN-γ, IL-17A) was analyzed.

2 Results

2.1 1st publication

Title of publication:

Canine tissue-associated CD4$^+$CD8α$^+$ double-positive T cells are an activated T cell subpopulation with heterogeneous functional potential

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- practical experimental work
- data acquisition
- data analysis and interpretation
- manuscript writing

Doris Bismarck: manuscript revision

Martina Protschka: assistance with practical experimental work, manuscript revision

Gabriele Köhler: histopathological examination of samples

Peter F. Moore: supply of selected antibodies

Mathias Büttner: support to experimental design, manuscript revision

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- conceptualization
- support to experimental design and data interpretation
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Maria Eschke:

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RESEARCH ARTICLE

Canine tissue-associated $CD4^+CD8\alpha^+$ double-positive T cells are an activated T cell subpopulation with heterogeneous functional potential

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Abstract

Canine $CD4^+CD8\alpha^+$ double-positive (dp) T cells of peripheral blood are a unique effector memory T cell subpopulation characterized by an increased expression of activation markers in comparison with conventional $CD4^+$ or $CD8\alpha^+$ single-positive (sp) T cells. In this study, we investigated $CD4^+CD8\alpha^+$ dp T cells in secondary lymphatic organs (i.e. mesenteric and tracheobronchial lymph nodes, spleen, Peyer's patches) and non-lymphatic tissues (i.e. lung and epithelium of the small intestine) within a homogeneous group of healthy Beagle dogs by multi-color flow cytometry. The aim of this systematic analysis was to identify the tissue-specific localization and characteristics of this distinct T cell subpopulation. Our results revealed a mature extrathymic $CD1a^-CD4^+CD8\alpha^+$ dp T cell population in all analyzed organs, with highest frequencies within Peyer's patches. Constitutive expression of the activation marker CD25 is a feature of many $CD4^+CD8\alpha^+$ dp T cells independent of their localization and points to an effector phenotype. A proportion of lymph node $CD4^+CD8\alpha^+$ dp T cells is $FoxP3^+$ indicating regulatory potential. Within the intestinal environment, the cytotoxic marker granzyme B is expressed by $CD4^+CD8\alpha^+$ dp intraepithelial lymphocytes. In addition, a fraction of $CD4^+CD8\alpha^+$ dp intraepithelial lymphocytes and of mesenteric lymph node $CD4^+CD8\alpha^+$ dp T cells is $TCR\gamma\delta^+$. However, the main T cell receptor of all tissue-associated $CD4^+CD8\alpha^+$ dp T cells could be identified as TCRαβ. Interestingly, the majority of the $CD4^+CD8\alpha^+$ dp T cell subpopulation expresses the unconventional CD8αα homodimer, in contrast to $CD8\alpha^+$ sp T cells, and $CD4^+CD8\alpha^+$ dp thymocytes which are mainly $CD8\alpha\beta^+$. The presented data provide the basis for a functional analysis of tissue-specific $CD4^+CD8\alpha^+$ dp T cells to elucidate their role in health and disease of dogs.

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Introduction

Extrathymic $CD4^+CD8\alpha^+$ double-positive (dp) T cells are a mature T cell subpopulation distinct from conventional $CD4^+$ single-positive (sp) T helper and $CD8\alpha^+$ sp cytotoxic T cells known to occur in different species, e.g. swine, humans, monkeys, mice, rats, and chicken [1–9]. Canine $CD4^+CD8\alpha^+$ dp T cells within peripheral blood mononuclear cells (PBMC) were first described around ten years ago [10–13]. To date, our group was able to characterize this unconventional T cell subpopulation in peripheral blood as mature T cell receptor (TCR) $\alpha\beta^+CD1a^-$ effector memory T cells [14,15]. $CD4^+CD8\alpha^+$ dp T cells can develop from both, $CD4^+$ sp as well as from $CD8\alpha^+$ sp T cells upon *in vitro* stimulation, but $CD4^+$ sp T cells are the more potent progenitors [16]. Furthermore, canine $CD4^+CD8\alpha^+$ dp T cells of the peripheral blood can be divided into three different subsets, i.e. $CD4^{dim}CD8\alpha^{bright}$, $CD4^{bright}CD8\alpha^{bright}$, and $CD4^{bright}CD8\alpha^{dim}$, which differ in phenotype and functional features. The $CD4^{dim}CD8\alpha^{bright}$ subset expresses the CD8αβ heterodimer, whereas most cells of the other two subsets express the unconventional CD8αα homodimer [14,15]. In contrast to CD8αβ, CD8αα does not work as a TCR co-receptor, but was shown to negatively regulate the activation of T cells [17].

Another unique feature of the total $CD4^+CD8\alpha^+$ dp T cell subpopulation of PBMC is their significantly higher expression level of CD25 and of MHC-II in comparison to their $CD4^+$ and $CD8\alpha^+$ sp counterparts, suggesting a high level of activation and an important immunological potential [15]. In fact, human $CD4^+CD8\alpha^+$ dp T cells are associated with autoimmune diseases [18], infections, e.g. human immunodeficiency virus (HIV) infections [19], inflammatory bowel disease [20], atopic dermatitis [21], and breast cancer [22]. The latter are also common diseases of dogs, making the dog an interesting animal model for common human health disorders. Very recently, increased frequencies of $CD4^+CD8\alpha^+$ dp T cells could be found in blood and spleen of dogs infected with *Ehrlichia chaffeensis* [23]. Additionally, $CD4^+CD8\alpha^+$ dp T cells were reported to increase in the context of canine leishmaniasis [24], pointing to an important role during canine immune responses.

However, still the developmental origin and the function of canine $CD4^+CD8\alpha^+$ dp T cells *in vivo* have not yet been clarified. For this purpose, reliable reference data in a homogeneous group of healthy animals including a comprehensive phenotypical and functional characterization not only from blood, but also from different lymphatic and non-lymphatic organs are required. Our group had the unique opportunity to study $CD4^+CD8\alpha^+$ dp T cells of tracheobronchial (tLN) and mesenteric (mLN) lymph nodes, spleen, Peyer's patches (PP), lung, and of small intestinal intraepithelial lymphocytes (IEL) from a standardized cohort of healthy Beagle dogs. As control, thymocytes of the same dogs were analyzed. The presented data allow a deeper insight into the characteristics of canine $CD4^+CD8\alpha^+$ dp T cells of secondary lymphatic and mucosal organs. Besides, the data will provide the basis for further functional analyses to elucidate the *in vivo* role of $CD4^+CD8\alpha^+$ dp T cells in dogs.

Materials and methods

Animals

Tissue samples were taken from healthy experimental Beagle dogs (Marshall Bioresources, North Rose, NY, USA, 12 dogs in total, six female, six male, age: 10–15 months) euthanized as control group of an animal experiment for preclinical drug development unrelated to our studies (approval number V54-19c 20/15-DA4/Anz.1004). All efforts were made to minimize suffering of the dogs. The physical health of all animals was confirmed by necropsy and histopathological examination. Directly after euthanasia, the dogs were fully bled and full

thickness sections from duodenum and jejunum, mesenteric (mLN) and tracheobronchial (tLN) lymph nodes, lung, spleen, and thymus were collected for further processing (n = 10 for mLN, Peyer's patches, lung, spleen, and thymus, n = 9 for tLN, n = 6 for intraepithelial lymphocytes of the small intestine).

Generation of single cell suspensions of spleen, thymus and lymph nodes

1 x 1 x 0.5 cm^3 pieces of spleen, thymic tissue, mLN, and tLN (size variable) were minced, passed through a 100 μm nylon cell strainer (BD Biosciences, Heidelberg, Germany) and resuspended in phosphate buffered saline (PBS). The cell suspensions were centrifuged at 500 x g for 10 min at 4˚C. The splenic cells were treated with erythrocyte lysis buffer (150 mM NH_4Cl, 8 mM $KHCO_3$, 2 mM EDTA; pH 7) for 5 min at room temperature (RT) and the lysis reaction was stopped with PBS containing 3% fetal bovine serum (FBS, Thermo Fisher Scientific, Carlsbad, USA). Again, the splenic cell suspension was centrifuged at 500 x g for 10 min at 4˚C and resuspended in PBS. The cell numbers were determined with a microscope using a hemocytometer (Laboroptik, Lancing, UK) and trypan blue (Sigma-Aldrich, Taufkirchen, Germany).

Isolation of lung leukocytes

Tissue pieces of lung (3 x 3 x 0.5 cm^3) were minced and digested for 30 min at 37˚C in RPMI1640 medium (Biochrom, Berlin, Germany) supplemented with DNase I (111 U/ml; Sigma-Aldrich, Taufkirchen, Germany), Collagenase D (0.7 mg/ml; Roche Diagnostics Deutschland GmbH, Mannheim, Germany), and 1 mM sodium pyruvate (AppliChem, Darmstadt, Germany) with slow agitation. Single cell suspensions were obtained by passaging digested lung tissue through 100 μm cell strainers (BD Biosciences, Heidelberg, Germany). Subsequently, erythrocytes were lysed as described above. For enrichment of leukocytes, the cells were resuspended in 70% Percoll (GE Healthcare, Uppsala, Sweden) diluted in RPMI 1640 medium and layered under 30% Percoll. Following density gradient centrifugation (400 x g, 20 min, RT), leukocytes were recovered from the interphase, resuspended in IMDM medium (PAN-Biotech, Aidenbach, Germany) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (both purchased from PAA Laboratories), and 10% FBS (Thermo Fisher Scientific, Carlsbad, USA). The cell number was determined as described above.

Isolation of lymphocytes from Peyer's patches and from the small intestinal epithelium

A protocol to isolate lymphocytes from Peyer's patches (PP) was adapted from a method described for pigs by Solano-Aguilar *et al.* [25]. Briefly, PP were collected from the duodenum and jejunum and immediately washed in ice-cold washing buffer consisting of Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (PAN-Biotech, Aidenbach, Germany), 10 mM HEPES (Carl Roth, Karlsruhe, Germany) and 2% FBS (Thermo Fisher Scientific, Carlsbad, USA). After that, the intestinal epithelium was removed by incubating the pieces 3 x 30 min in washing buffer containing 2 mM 1,4-Dithioerythritol (DTE, Sigma-Aldrich, Taufkirchen, Germany), and 0.5 mM EDTA (Carl Roth, Karlsruhe, Germany) at 37˚C under continuous stirring. The supernatant was discarded, whereas the tissue was minced with scalpels, dissociated by the gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), and passaged through a 100 μm cell strainer (BD Biosciences, Heidelberg, Germany). Lymphocytes were separated from enterocytes by 40%/70% Percoll density gradient centrifugation (900 x g, 20 min, RT), harvested at the interphase, and washed in RPMI1640 medium

(Biochrom, Berlin, Germany) containing 5% FBS and 50 μg/ml Gentamicin (Sigma-Aldrich, Taufkirchen, Germany).

For isolation of intraepithelial lymphocytes (IEL), duodenum and proximal jejunum were cut into 0.5 x 0.5 cm^2 pieces under exclusion of PP and washed in ice-cold washing buffer (see above). The epithelium was separated from the lamina propria as described for PP. After the incubation process, the supernatant containing the epithelium was first filtered through a 100 μm cell strainer, and in a second step through a 70 μm cell strainer (SPL Life Sciences, Pocheon, South Korea). The mucus was removed by washing the cell pellet in a 25% Percoll solution (900 x g, 30 min, RT). Lymphocytes were separated from intestinal epithelial cells by 25%/47%/66% Percoll density gradient centrifugation (900 x g, 20 min, RT) and collected from the lymphocyte band. For both PP and IEL, erythrocytes were lysed and the cell number was determined as described above.

To confirm the appropriate separation of the epithelium from PP resp. the lamina propria, control samples before and after treatment were fixed in 4% formalin (Carl Roth, Karlsruhe, Germany) and embedded in paraffin (Leica, Heidelberg, Germany) followed by a hematoxylin-eosin stain (Merck, Darmstadt, Germany) and microscopical examination of 4 μm sections (S1 Fig).

Flow cytometric analysis

To discriminate dead from viable cells, isolated cells from lymphatic and non-lymphatic organs were stained with fixable viability dye eFluor 780 (Thermo Fisher Scientific, Carlsbad, USA) according to the manufacturer's protocol. Prior to direct labeling with fluorochrome-conjugated antibodies, the cells were incubated with a mixture of heat-inactivated normal serum derived from dog, rat, and mouse (each 15% in PBS) to block unspecific binding of Fc receptors. In case of indirect labeling with a secondary goat anti-mouse antibody, Fc receptor blockade was performed with a mixture of heat-inactivated rat, dog and goat normal serum (each 15% in PBS). Primary antibodies used for flow cytometric staining are summarized in Table 1. For definition of positive and negative populations during the analysis, fluorescence minus one (FMO) controls were included in the experiments. FMO controls contain all specific antibodies of the staining panel, except the one of interest which is replaced by its isotype control. The corresponding isotype control antibodies were purchased from Thermo Fisher Scientific (Carlsbad, USA) or Biolegend (San Diego, USA), respectively. The cells were incubated with primary antibodies for 15 min in the dark on ice. Canine CD1a, CD8β, TCRαβ, and TCRγδ were detected by the use of a PerCP/Cy5.5-conjugated goat-anti-mouse IgG secondary antibody (Biolegend, San Diego, USA). In case of exclusive surface staining, the cells were fixed with 2% paraformaldehyde (Sigma-Aldrich, Taufkirchen, Germany) for 15 min in the dark on ice prior to the analysis. For intracellular detection of granzyme B and FoxP3, the FoxP3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Carlsbad, USA) was used according to the manufacturer's protocol. The cells were acquired with a BD LSR Fortessa flow cytometer (Becton Dickinson, Heidelberg, Germany) and viable $CD5^+$ lymphocytes were analyzed using the FlowJo 10 software (Treestar Inc., Ashland, OR, USA) after doublet exclusion. See supporting information for gating strategy (S2 Fig).

Statistical analysis

The evaluation of statistical significance was performed using Graph Pad Prism 5.01 software (San Diego, CA, USA). Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lilliefor p value) was applied to test for normality. In case of normal distribution of all data sets mean values are presented within one graph. The unpaired Student's t-test (two-tailed) was used to compare

Table 1. Primary antibodies used for flow cytometry.

Antigen	Clone	Species Reactivity	Isotype	Formats
CD5	YKIX322.3[a]	dog	Rat IgG2a	PE PerCP-eFluor 710
CD4	YKIX302.9[a, b]	dog	Rat IgG2a	FITC, APC Pacific Blue
CD8α	YCATE55.9[a, b]	dog	Rat IgG1	APC Pacific Blue Alexa Fluor 647
CD1a	CA13.9H11[c]	dog	Mouse IgG1	Hybridoma supernatant
CD8β	CA15.4G2[c]	dog	Mouse IgG1	Hybridoma supernatant Biotin
TCRγδ	CA20.8H1[c]	dog	Mouse IgG1	Hybridoma supernatant
TCRαβ	CA15.8G7[c]	dog	Mouse IgG1	Hybridoma supernatant
CD25	P4A10[a]	dog	Mouse IgG1	PE
FoxP3	FJK-16s[a]	mouse/rat published crossreactivity with dog [14,26,27]	Rat IgG2a	FITC
Granzyme B	GB11[d]	human/mouse published crossreactivity with dog [14]	Mouse IgG1	FITC

[a] Thermo Fisher Scientific, Carlsbad, USA

[b] Bio-Rad, Munich, Germany

[c] Leukocyte Antigen Biology Laboratory, VM PMI, School of Veterinary Medicine, University of California, Davis, USA

[d] Biolegend, San Diego, USA

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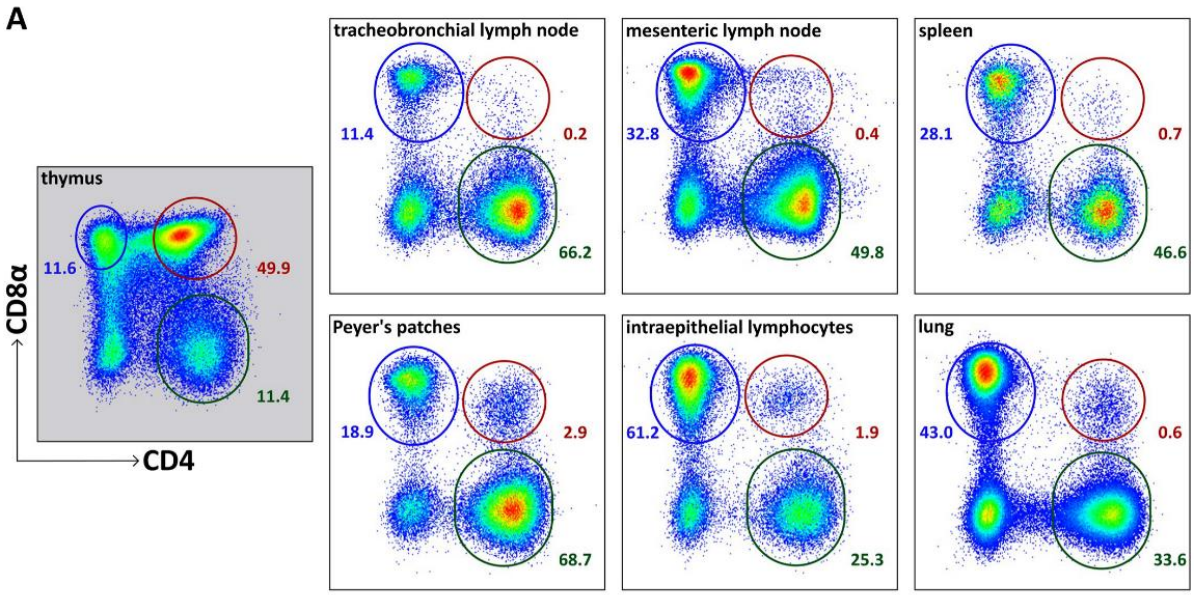
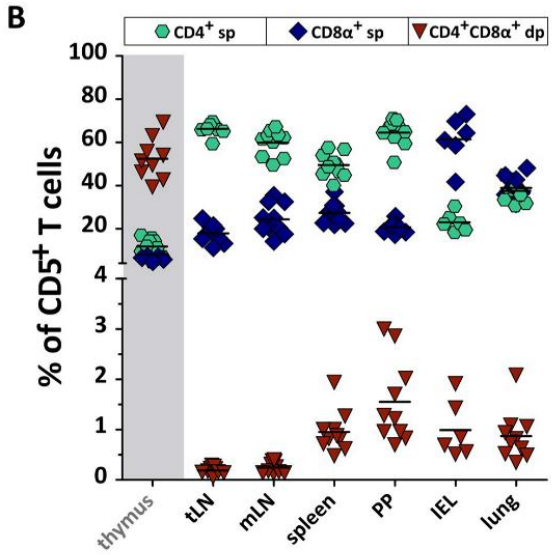
two groups whereas differences between more than two groups were analyzed by One-way analysis of variance (ANOVA) with Bonferroni post hoc test. If graphs include nonparametric data, the median is depicted. Multiple comparisons were performed by use of the Kruskal-Wallis H test with Dunn's post test. If only two groups were included in the analysis, the Mann-Whitney U test (two-tailed) was used. The level of confidence for significance is depicted in figure legends.

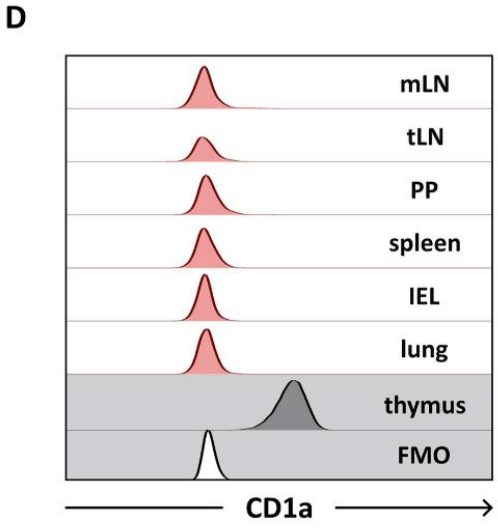
Results

Highest frequencies of mature CD4$^+$CD8α$^+$ double-positive T cells are present in Peyer's patches compared to other secondary lymphatic and non-lymphatic organs of healthy dogs

To gain an in depth understanding of canine CD4$^+$CD8α$^+$ double-positive (dp) T cells, a further characterization in other tissues than PBMC is required. Here we analyzed different lymphatic and non-lymphatic organs, i.e. tracheobronchial (tLN) and mesenteric (mLN) lymph nodes, spleen, Peyer's patches (PP), lung, and small intestinal intraepithelial lymphocytes (IEL) from a homogeneous cohort of healthy Beagle dogs. In all analyzed organs a fraction of CD4$^+$CD8α$^+$ dp T cells could be detected (Fig 1A). Our analysis revealed significant differences concerning the CD4$^+$CD8α$^+$ dp T cell frequencies depending on the organ, with highest frequencies in PP (1.6% on average) and lowest in tLN (0.2% on average) (Fig 1B and 1C). It is known that canine CD4$^+$CD8α$^+$ dp T cells of the peripheral blood form a heterogeneous cell population which can be divided into three different subsets, namely CD4^{bright}CD8α^{dim}, CD4^{bright}CD8α^{bright}, and CD4^{dim}CD8α^{bright} [15]. In contrast to PBMC, the CD4$^+$CD8α$^+$ dp T cells of spleen, PP, IEL and lung constitute one homogeneous population which cannot be divided into different subsets. In tLN and mLN a more heterogeneous, but very small







C

% of CD4+CD8α+dp T cells: Peyer's patches (PP) vs. indicated organs

	tLN	mLN	spleen	IEL	lung
PP	***	***	*	n. s.	*

Fig 1. Mature CD4+CD8α+ double-positive (dp) T cells are present in secondary lymphatic organs as well as in non-lymphatic tissues of healthy dogs with highest frequencies in Peyer's patches. (A) The frequency of canine CD4+ single-positive (sp), CD8α+ sp, and CD4+CD8α+ dp T cells in different organs was analyzed by flow cytometry. Shown are representative pseudocolor plots with numbers indicating percentages. Organs of 6-10 dogs in total have been analyzed in two independent experiments. Thymus was used as control (grey background). Only living non-doublet CD5+ T cells were included in the analysis. The gating strategy is shown in supporting S2 Fig. (B) Quantification of CD4+ sp (green hexagons), CD8α+ sp (blue diamonds), and CD4+CD8α+ dp (red triangles) T cells in canine tracheobronchial lymph nodes (tLN), mesenteric lymph nodes (mLN), spleen, Peyer's patches (PP), intraepithelial lymphocytes of the small intestine (IEL), and lung is depicted. Thymus served as control (grey background). Pooled data of two independent experiments are shown. Each dot represents one individual dog, the horizontal bars indicate mean values. For clarity reasons, a statistical analysis (One-way ANOVA with Bonferroni's Multiple

Comparison Test, * $p < 0.05$, *** $p < 0.001$, n. s.: not significant) is shown in (C). (D) Tissue-associated canine CD4+CD8α+ dp T cells are mature T cells lacking the thymic marker CD1a (red histograms). As control, thymus (grey histogram) was analyzed for CD1a expression with the appropriate fluorescence minus one (FMO) control (white histogram). Results of one representative dog are depicted. Organs of 4–10 dogs in total have been analyzed in two independent experiments.

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population of CD4+CD8α+ dp T cells lacking the $CD4^{bright}CD8\alpha^{bright}$ subset is present (Fig 1A). Yet a subdivision into subsets would have restricted further analyses due to low cellular numbers. As expected, the CD4+CD8α+ dp fraction in the thymus serving as control is predominating (Fig 1B). These immature thymocytes, however, express the thymic marker CD1a, which distinguishes them from the CD1a-CD4+CD8α+ dp T cells in secondary lymphatic organs, lung and intestine (Fig 1D) and from previously reported peripheral blood CD1a-CD4+CD8α+ dp T cells [15]. Consequently, the extrathymic CD4+CD8α+ dp T cells exhibit a mature phenotype.

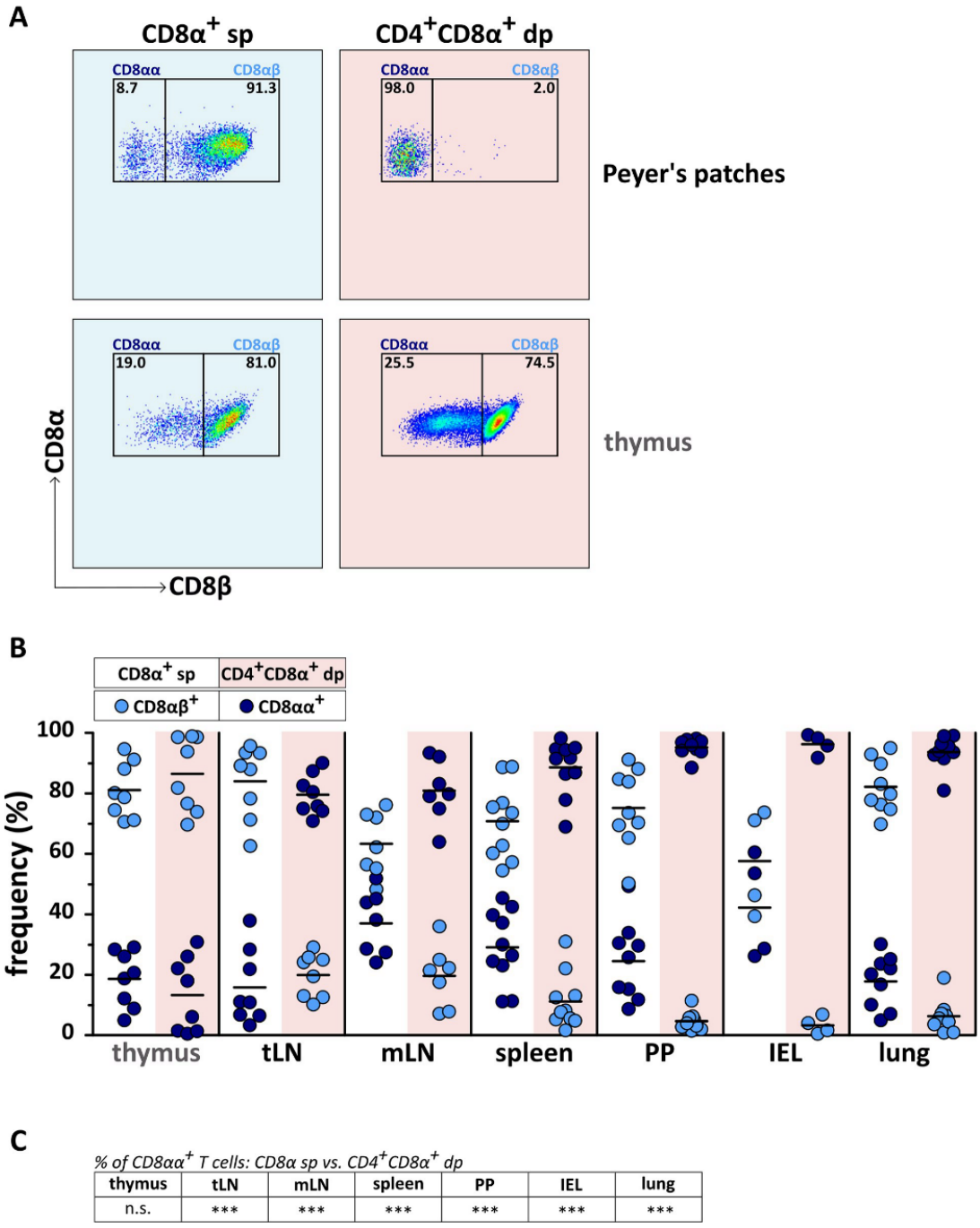
CD4+CD8α+ double-positive T cells of secondary lymphatic and non-lymphatic organs differ from CD8α+ single-positive (sp) T cells and CD4+CD8α+ dp thymocytes in their CD8αα expression, and a high proportion is TCRαβ+

CD8+ T cells express their CD8 surface receptor either as a CD8αβ heterodimer or as a CD8αα homodimer. CD8αβ promotes the activation of T cells with low-affinity TCR, whereas CD8αα increases the activation threshold [17,28]. Therefore, we investigated the composition of the CD8 receptor of mature CD4+CD8α+ dp extrathymic T cells and compared it with their CD8**α**+ sp counterparts, and with immature CD4+CD8**α**+ dp thymocytes. In all analyzed organs, as expected, the majority of the CD8**α**+ sp subpopulation is CD8β+ indicating expression of the CD8αβ heterodimer (Fig 2A and 2B). As in blood, only a small fraction of CD8α+ sp T cells does not express CD8β, equivalent with a CD8αα phenotype. In the thymus, both the CD8α+ sp and the CD4+CD8α+ dp fraction are predominantly CD8αβ+. In marked contrast to this, the main CD8 receptor of the mature extrathymic CD4+CD8α+ dp T cells is the CD8αα homodimer, whereas the CD8αβ heterodimer only comprises a small proportion (Fig 2A–2C), underlining the distinct features of this T cell subpopulation.

As one subpopulation of unconventional CD8αα+ sp T cells of IEL from small intestine of dogs has been published to express the T cell receptor γδ (TCRγδ) [29], we were interested in the TCRγδ expression of CD4+CD8α+ dp extrathymic T cells in peripheral tissues. Despite their CD4+CD8αα+ phenotype, TCRγδ+ dp T cells are nearly absent in lung, spleen, mLN, PP, and thymus (Fig 3A). This result pointed to the expression of TCRαβ and was confirmed by direct TCRαβ staining of splenic CD4+CD8α+ dp T cells of the same dogs (S3 Fig). Due to limited cell number, tLN could not be included in TCRγδ analysis. Interestingly, although with a high inter-individual variation, only within IEL (median ~6%) and mLN (median ~1%) a proportion of CD4+CD8α+ dp T cells was found to be TCRγδ+ (Fig 3A and 3B). This is in contrast to CD8α+ sp T cells from which up to 5% express TCRγδ in all analyzed organs (Fig 3B).

A high proportion of tissue-associated mature CD4+CD8α+ dp T cells expresses the activation marker CD25

CD4+CD8α+ dp thymocytes are known as an immature intermediate stage during T cell development. Accordingly, these cells lack expression of activation markers such as CD25 [30–32], which we could confirm in our study (Fig 4A and 4B). In contrast, mature CD4+CD8α+ dp T cells of secondary lymphatic and non-lymphatic tissues constitutively show high frequencies



C

% of CD8αα+ T cells: CD8α sp vs. CD4+CD8α+ dp

thymus	tLN	mLN	spleen	PP	IEL	lung
n.s.	***	***	***	***	***	***

Fig 2. The majority of canine CD4+CD8α+ double-positive (dp) T cells in secondary lymphatic and non-lymphatic organs is characterized by expression of the CD8αα homodimer. (A) CD4+CD8α+ dp T cells were analyzed by flow cytometry for the expression of CD8αα and CD8αβ in comparison to CD8α+ single-positive (sp) T cells. The pseudocolor plots of Peyer's patches stand representative for all secondary lymphatic and non-lymphatic organs in this study. Thymocytes are shown for control. The numbers in the pseudocolor plots imply percentages. Organs of 4–10 dogs in total have been analyzed in two independent experiments. The CD8α+CD8β- population indicates a CD8αα homodimer, the

CD8α+CD8β+ population a CD8αβ heterodimer. (B) Proportions of CD8αα (dark blue dots) vs. CD8αβ (light blue dots) expression of CD8α+ sp (white background) and mature CD4+CD8α+ dp T cells (red background) in tracheobronchial lymph node (tLN), mesenteric lymph node (mLN), spleen, Peyer's patches (PP), intraepithelial lymphocytes of the small intestine (IEL), and lung with thymus as control were quantified. Pooled data of two independent experiments are shown. Each dot represents one individual dog, the horizontal bars indicate mean values. Differences in percentages of CD8αα+ cells among CD4+CD8α+ dp and CD8α+ sp T cells were analyzed by unpaired Student's t-test (two-tailed; *** $p < 0.001$) and are summarized in (C).

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of CD25 expression (Fig 4A–4C), in line with our findings about CD4+CD8α+ dp T cells of the peripheral blood [14,15] and corresponding to an activated phenotype. Compared to their single-positive counterparts, frequencies of CD25+ T cells among CD4+CD8α+ dp T cells were significantly higher in all analyzed secondary lymphatic organs (tLN, mLN, spleen, PP). Interestingly, despite the high abundance of CD25+ T cells within the CD4+CD8α+ dp subpopulation, the IEL of the small intestine revealed no significant difference between CD4+ or CD8α+ sp and CD4+CD8α+ dp T cells concerning their CD25 expression (Fig 4B).

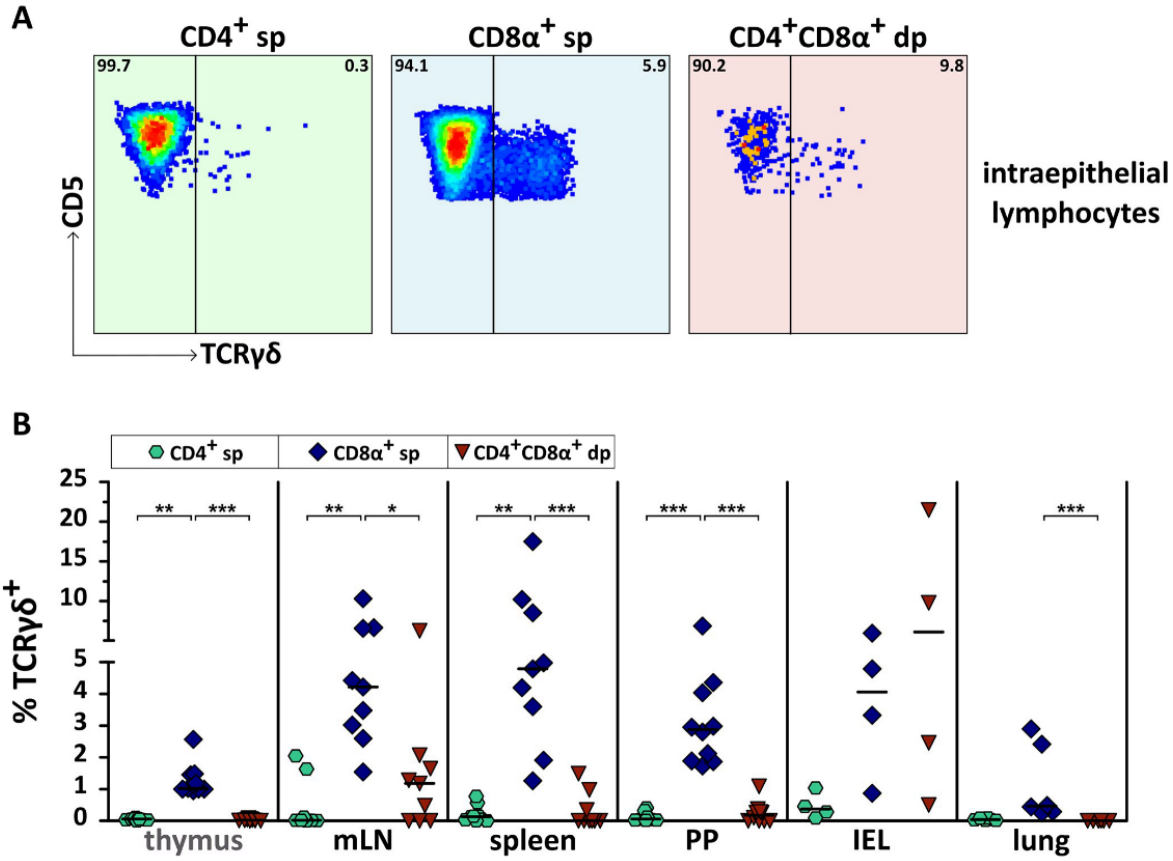

Fig 3. Canine CD4+CD8α+ double-positive (dp) T cells in secondary lymphatic and non-lymphatic organs are mainly TCRγδ-negative. (A) Shown are representative results of T cell receptor γδ (TCRγδ) expression in CD4+CD8α+ dp, CD4+ sp, and CD8α+ sp T cells among intraepithelial lymphocytes of the small intestine (IEL). The numbers in the flow cytometry plots indicate percentages. (B) Quantification of the frequency of TCRγδ+ cells among CD4+ sp (green hexagons), CD8α+ sp (blue diamonds), and CD4+CD8α+ dp (red triangles) T cells in mesenteric lymph node (mLN), spleen, Peyer's patches (PP), IEL, and lung is shown. Thymus is depicted for control. Pooled data of two independent experiments are shown. Each dot represents one individual dog, the horizontal bars indicate median values. Statistical analysis was performed by One-way ANOVA with Dunn's Multiple Comparison Test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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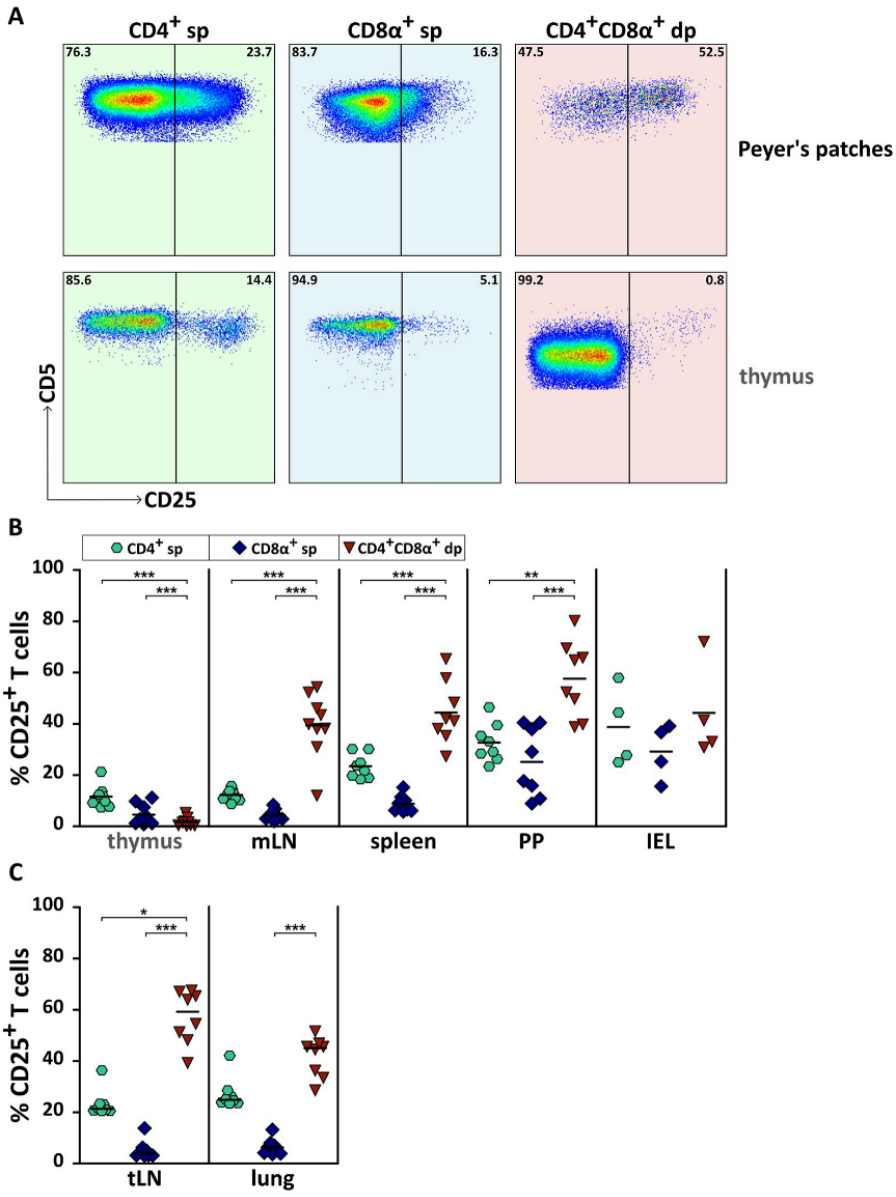

Fig 4. Canine CD4$^+$CD8α$^+$ double-positive (dp) T cells of secondary lymphatic and non-lymphatic organs constitutively express CD25 in contrast to CD4$^+$CD8α$^+$ dp thymocytes. (A) The expression of CD25 on CD4$^+$ single-positive (sp), CD8α$^+$ sp and CD4$^+$CD8α$^+$ dp T cells was analyzed by flow cytometry. Shown are representative data of Peyer's patches in comparison to thymus. The numbers in the pseudocolor plots indicate percentages. Two independent experiments have been performed including organs of 4–10 dogs in total. (B + C) Pooled data of CD25 expression on CD4$^+$ sp (green hexagons), CD8α$^+$ sp (blue diamonds), and CD4$^+$CD8α$^+$ dp (red triangles) T cells in indicated organs are depicted. (B) The graphs include normally distributed data values, each symbol represents one individual dog. The horizontal bars indicate mean values. Statistical analysis was performed by One-way ANOVA with Bonferroni's Multiple Comparison Test (** $p < 0.01$, *** $p < 0.001$). (C) The graphs show non-normally distributed data values, each symbol represents one individual dog. The horizontal bars indicate median values. Statistical analysis was performed by One-way ANOVA with Dunn's Multiple Comparison Test (* $p < 0.05$, *** $p < 0.001$).

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CD4+CD8α+ dp T cells in lymph nodes contain FoxP3+ regulatory T cells

Given the high frequencies of CD25 in dp T cells, we analyzed the transcription factor forkhead box P3 (FoxP3) which is specific for regulatory T cells (Treg) [33]. As expected, immature CD4+CD8α+ dp thymocytes are FoxP3- (Fig 5A and 5C). FoxP3 expression of mature CD4+CD8α+ dp T cells of secondary lymphatic and non-lymphatic organs is heterogeneous. In all analyzed organs except lymph nodes, only low proportions of CD4+CD8α+FoxP3+ dp T cells (≤ 3%) were detected. In lymph nodes, however, percentages reach ~10% in mLN and ~20% in tLN. Furthermore, these percentages are even significantly higher than are those of mLN and tLN CD4+FoxP3+ sp T cells (Fig 5C). Although the CD4+CD8α+ dp T cell subpopulation in lymph nodes is rather small (Fig 1), the distribution of fluorescence (i.e. mean fluorescence intensity, MFI) of the FoxP3+ events is comparable to the positive control (i. e. CD4+ sp T cells), indicating a valid signal [34]. Furthermore, the intracellular expression of FoxP3 in lymph nodes correlates with the degree of surface expression of CD25 on CD4+CD8α+ dp T cells (S4 Fig). Taken together, these results point to a regulatory potential of CD4+CD8α+ dp T cells in lymph nodes.

IEL CD4+CD8α+ dp T cells express the cytotoxicity marker granzyme B

Finally, we investigated whether CD4+CD8α+ dp T cells of lymphatic and non-lymphatic tissues show features of cytotoxic T cells by analyzing their intracellular granzyme B expression. As most IEL are known to exhibit constitutive cytolytic capacity in human and mice [28,35], we focused on the intestinal environment. While granzyme B expression in CD4+CD8α+ dp T cells of gut-associated lymphoid tissue (GALT) is absent (mLN 0%) resp. low (PP 1.6% on average), a considerable proportion (15% on average) of IEL CD4+CD8α+ dp T cells is granzyme B+ (Fig 6). In accordance with literature [36,37], some CD4+ sp T cells expressing granzyme B are also present in the small intestinal epithelium. As expected, a high proportion of CD8α+ sp T cells expresses granzyme B within IEL. Furthermore, granzyme B+ CD8α+ sp T cells are present in PP but not in mLN.

Discussion

Canine CD4+CD8α+ double-positive (dp) T cells of peripheral blood are a heterogeneous effector memory T cell subpopulation suggesting an important role in immune response by their constitutively high activation [14,15]. Here we provide the first systematic characterization of CD4+CD8α+ dp T cells in different tissues within one homogeneous group of healthy Beagle dogs. This reveals insight into potential induction and/or effector sites of this unique T cell subpopulation and is a prerequisite for subsequent functional analyses. Our comprehensive study shows that highest frequencies of CD4+CD8α+ dp T cells are present in Peyer's patches (PP) compared to tracheobronchial and mesenteric lymph nodes (LN), spleen, intraepithelial lymphocytes of the small intestine (IEL), and lung. Former reports about canine CD4+CD8α+ dp T cells in LN [24,38], bone marrow [24], spleen [23], and IEL [39] are limited by either a low number [23] or by heterogeneity in breed, age, and/or health status of dogs [24,38,39]. Moreover, a detailed characterization of these cells was still missing. We could demonstrate that the majority of CD4+CD8α+ dp T cells in dogs is TCRαβ+, and, interestingly, up to 96% on average (IEL, PP) express the unconventional CD8αα homodimer. CD8αα was formerly thought to be a functional homolog of the MHC class I binding TCR co-receptor CD8αβ [40]. However, the CD8β chain has been identified as key molecule for CD8-dependent TCR function [41]. Transient co-expression of CD8αα on CD8αβ+ T cells, in contrast, results in decrease of TCR signal transduction inducing a higher activation threshold proportional to the expression level of CD8αα. Nevertheless, increased antigen stimulation can

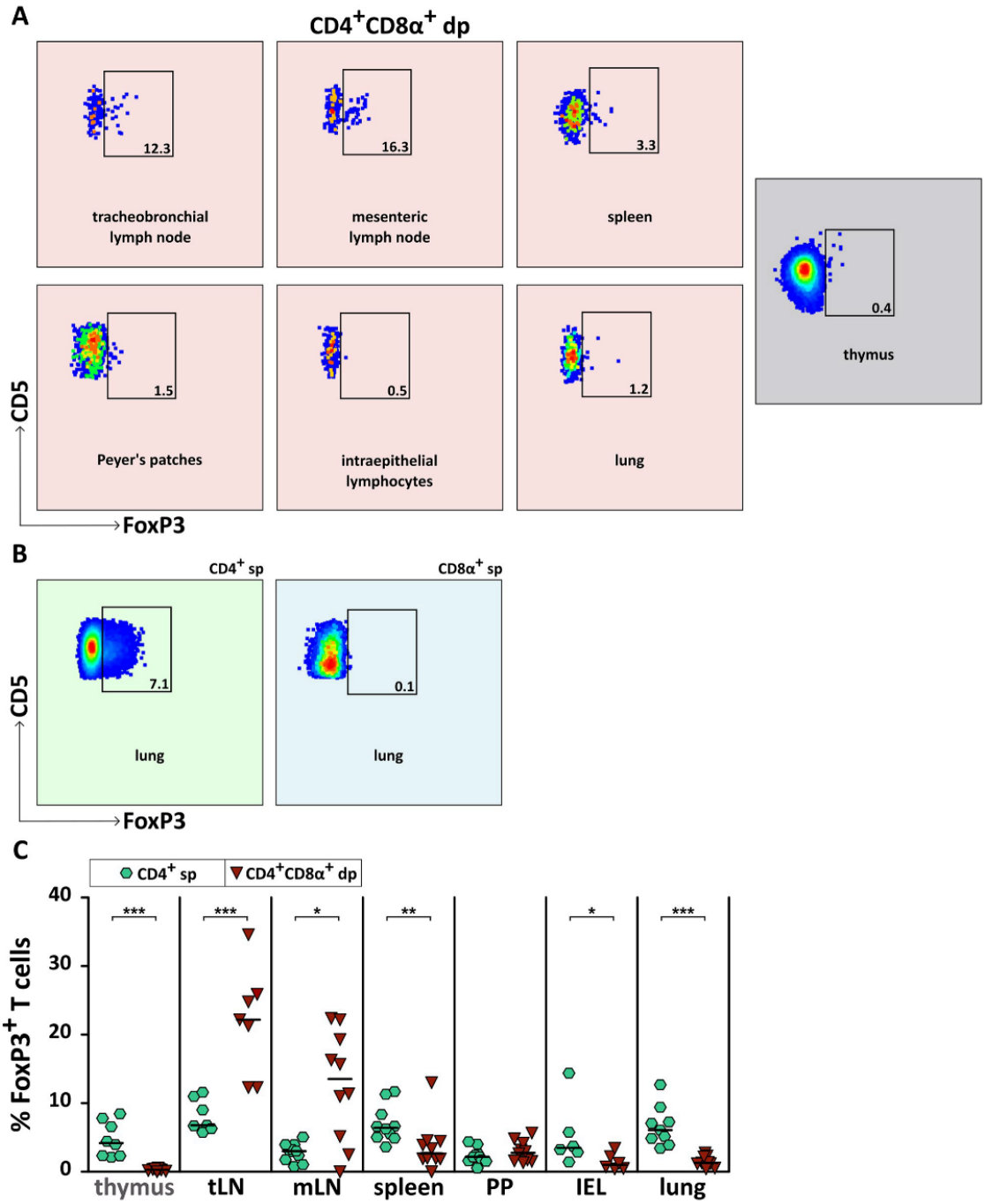

Fig 5. A proportion of lymph node CD4$^+$CD8α$^+$ double-positive (dp) T cells comprises FoxP3$^+$ regulatory T cells. (A) Representative results of FoxP3 expression in CD4$^+$CD8α$^+$ dp T cells of secondary lymphatic and non-lymphatic organs, and of CD4$^+$CD8α$^+$ dp thymocytes are shown. Two independent experiments with n = 4–10 dogs in total were performed. The numbers in the flow cytometry plots indicate percentages. Appropriate gating was confirmed by internal negative (CD8α$^+$ single-positive (sp) T cells) and positive (CD4$^+$ sp T cells) controls (B). Presented is the FoxP3 expression of CD4$^+$ sp and CD8α$^+$ sp T cells in canine lung being representative for all analyzed organs. (C) Proportions of FoxP3 expression of

CD4+ sp (green hexagons) and CD4+CD8α+ dp (red triangles) T cells were analyzed in indicated organs (tLN: tracheobronchial lymph node, mLN: mesenteric lymph node, PP: Peyer's patches, IEL: intraepithelial lymphocytes of the small intestine). Each symbol represents one individual dog, the horizontal bars indicate median values. The Mann-Whitney U test was performed to check for statistical significance between CD4+ sp and CD4+CD8α+ dp T cells (two-tailed; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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overcome the CD8αα repressor function [17,42]. CD4+CD8α+ dp T cells are characterized by high frequencies of CD25 expression, the IL-2 receptor α-chain (IL-2Rα) which constitutes the high-affinity IL-2 receptor in combination with IL-2Rβ and IL-2Rγ. The high-affinity IL-2 receptor enables rapid and effective proliferation of T cells [43]. As CD8αα has been shown to repress T cell activation [17,42], it might thus contribute to adequate induction of CD4+CD8α+ dp T cell effector functions only upon high antigenic stimulation and thereby prevent excessive immune responses. However, the function of CD8αα on CD4+ single-positive (sp) T cells has not yet been clarified and needs further investigation.

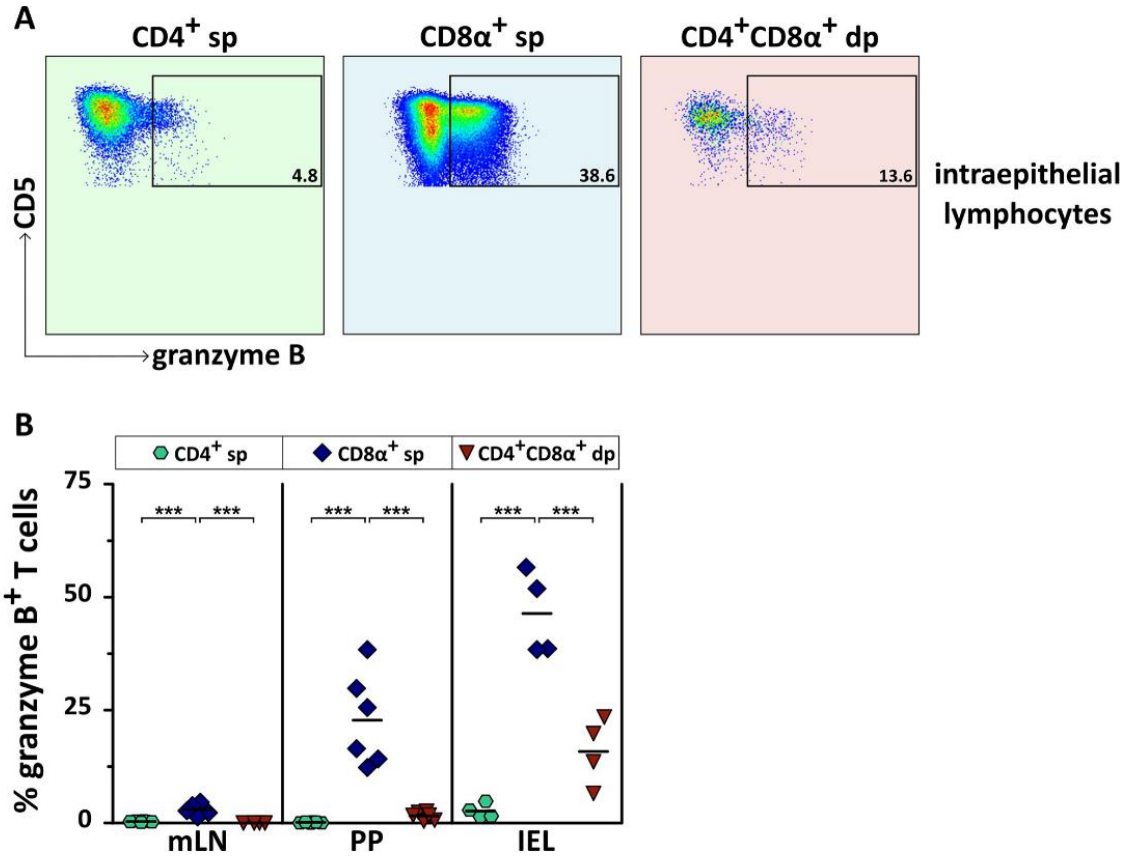

Fig 6. CD4+CD8α+ double-positive (dp) T cells of the small intestinal epithelium express the cytotoxic molecule granzyme B. (A) Representative results of granzyme B expression in CD4+CD8α+ dp T cells of small intestinal intraepithelial lymphocytes (IEL) are shown in comparison to CD4+ single-positive (sp) and CD8α+ sp T cells. The numbers in the flow cytometry plots indicate percentages. (B) Quantification of the frequency of granzyme B+ cells among CD4+ sp (green hexagons), CD8α+ sp (blue diamonds), and CD4+CD8α+ dp (red triangles) T cells in mesenteric lymph node (mLN), Peyer's patches (PP) and IEL is depicted. Each symbol represents one individual dog, the horizontal bars indicate mean values. Statistical analysis was performed by One-way analysis of variance (ANOVA) with Bonferroni post hoc test (*** $p < 0.001$).

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As demonstrated in previous *in vitro* studies, based on oligoclonal resp. polyclonal stimulation, canine PBMC $CD4^+CD8\alpha^+$ dp T cells can develop from both, $CD8\alpha^+$ and $CD4^+$ sp T cells, the latter being the more potent progenitors. $CD4^+CD8\alpha^+$ dp T cells emerging from $CD4^+$ sp T cells express CD8αα, whereas $CD4^+CD8\alpha^+$ dp T cells developing from $CD8\alpha^+$ sp T cells express CD8αα or CD8αβ [16]. Thus, the predominance of the CD8αα homodimer might be related to the main origin from $CD4^+$ sp T cells. In dogs, mice and humans the CD8αα isoform is constitutively expressed by $TCR\alpha\beta^+$ and $TCR\gamma\delta^+CD8^+$ sp IEL [28,29,35, 44,45] In addition, CD8αα can be upregulated on murine $TCR\alpha\beta^+CD4^+$ IEL *in vivo* [46,47], and *in vitro* in the presence of TGF-β and retinoic acid (RA) [36,48]. Furthermore, RA is produced by dendritic cells of the gut-associated lymphoid tissue (GALT), including PP [49,50]. This, in combination with high antigenic exposure in the gut, might lead to the induction of more $CD4^+CD8\alpha\alpha^+$ dp T cells in PP and in the intestinal epithelium than in LN, where RA is also present [51], but the antigenic load and diversity are less abundant. A possible association with antigenic stimulation was also hypothesized for porcine dp T cells [2] that account for a larger proportion of total $CD4^+$ lymphocytes in mucosa-associated lymphoid tissues as compared to lymph nodes [3,52]. In mice, many $CD4^+$ IEL co-express CD8α [48,53], whereas $CD4^+$CD8α dp T cells are rare in secondary lymphatic organs [1,8,53,54]. In humans, a significant percentage of $CD4^+CD8\alpha^+$ dp T cells has been described in the lamina propria of the gut [55]. Overall, this tissue distribution is related to an immunoregulatory and/or immunosurveillance function of $CD4^+CD8\alpha^+$ dp T cells as suggested previously [2].

Interestingly, we found evidence that the expression pattern of functional markers on canine $CD4^+CD8\alpha^+$ dp T cells is tissue-dependent, indicating heterogeneous functional potential. We show that $CD4^+CD8\alpha^+$ dp T cells of spleen, PP, IEL, and lung are mainly $FoxP3^-$. However, this transcription factor specific for regulatory T cells reaches significant proportions within LN. In contrast, comparable numbers of $FoxP3^+CD4^+CD8\alpha^+$ dp T cells with regulatory potential are present in mLN, spleen, and thymus of pigs [56]. According to van Kaer *et al*., murine $CD4^+CD8\alpha^+$ T cells induced *in vitro* only transiently express FoxP3 [57]. In support of this, Sujino *et al*. could demonstrate *in vivo* that murine regulatory T cells of the intestinal *lamina propria* lose FoxP3 expression upon migration into the intestinal epithelium where they become $FoxP3^-$ regulatory $CD4^+CD8\alpha\alpha^+$ IEL [58]. Furthermore, human $CD4^+CD8\alpha\alpha^+$ dp T cells of the colonic lamina propria have been shown to represent a regulatory T cell subset despite the lack of FoxP3 expression [20]. This leads us to the hypothesis that, in addition to $FoxP3^+CD4^+CD8\alpha^+$ dp T cells in canine LN, $FoxP3^-CD4^+CD8\alpha^+$ dp T cells might possess regulatory properties. However, further experiments are necessary to verify this hypothesis. In addition to regulatory $CD4^+CD8\alpha^+$ dp IEL [58], murine $CD4^+CD8\alpha^+$ dp IEL with cytolytic activity [36] have been described indicating the presence of different functional subsets. Since we found evidence by granzyme B staining that canine IEL $CD4^+CD8\alpha^+$ dp T cells also partly exhibit cytotoxic potential, the spectrum of effector functions of these cells should be subject of further research.

Taken together, this study provides a comprehensive characterization of $CD4^+CD8\alpha^+$ dp T cells in lymphatic and non-lymphatic organs of a homogeneous cohort of healthy Beagle dogs. We define these tissue-associated $CD4^+CD8\alpha^+$ dp T cells as an activated, but possibly self-regulating (by CD8αα expression) T cell subpopulation with heterogeneous functional (i.e. regulatory or cytotoxic) potential depending on its localization. This lays the foundation for future work on the role of $CD4^+CD8\alpha^+$ dp T cells in various organ-specific diseases of dogs.

Supporting information

S1 Fig. The separation of the epithelium from Peyer's patches and small intestinal villi was confirmed by hematoxylin and eosin (H&E) staining before and after treatment with DTE/

EDTA. Representative H&E-stained sections of Peyer's patches (A) and small intestine (B) before DTE/EDTA treatment are shown. Normal villous architecture with intact epithelium is visible. After DTE/EDTA treatment, the epithelial layer is removed from Peyer's patches (C), and from the villi (D), respectively. For the latter this is displayed at higher magnifications in the insets (compare B + D).
(TIF)

S2 Fig. General gating strategy used in flow cytometric analyses. Representative pseudocolor plots of Peyer's patches are depicted to show the general gating strategy. After exclusion of dead cells (A), gating on lymphocytes was performed according to their forward and side scattering (FSC/SSC) properties (B). Following doublet-exclusion (C), only CD5$^+$ T cells were included into subsequent analyses (D).
(TIF)

S3 Fig. The absence of TCRγδ corresponds with the presence of TCRαβ on CD4$^+$CD8α$^+$ double-positive (dp) T cells. (A) The expression of TCRαβ and TCRγδ on splenic CD4$^+$CD8α$^+$ dp T cells was analyzed by flow cytometry. Shown are pseudocolor plots of one representative dog. (B) Proportions of TCRγδ (grey dots) vs. TCRαβ (black dots) expression of mature splenic CD4$^+$CD8α$^+$ dp T cells were quantified. Each dot represents one individual dog, the horizontal bars indicate mean values.
(TIF)

S4 Fig. In lymph nodes, FoxP3$^+$CD4$^+$CD8α$^+$ double-positive (dp) T cells are mainly CD25^{high}. (A) Mesenteric lymph node CD4$^+$CD8α$^+$ dp T cells were analyzed for CD25 and FoxP3 expression. Representative plots show the distribution of FoxP3$^+$ cells in CD25^{neg}, CD25^{dim} and CD25^{high} subpopulations. The frequency (B) and mean fluorescence intensity (MFI) (C) of FoxP3 expression in CD25^{neg}, CD25^{dim}, and CD25^{high} CD4$^+$CD8α$^+$ dp T cells in lymph nodes was quantified. Each symbol represents one individual dog, the horizontal bars indicate median values. Statistical analysis was performed by One-way ANOVA with Dunn's Multiple Comparison Test (** $p < 0.01$).
(TIF)

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Writing – original draft: Friederike V. Rabiger, Maria Eschke.

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References

1. Overgaard NH, Jung J-W, Steptoe RJ, Wells JW. CD4+/CD8+ double-positive T cells: more than just a developmental stage. J Leukoc Biol. 2015; 97: 31–38. https://doi.org/10.1189/jlb.1RU0814-382 PMID: 25360000
2. Zuckermann FA. Extrathymic CD4/CD8 double positive T cells. Vet Immunol Immunopathol. 1999; 72: 55–66. https://doi.org/10.1016/S0165-2427(99)00118-X PMID: 10614493
3. Saalmüller A, Reddehase MJ, Bühring HJ, Jonjić S, Koszinowski UH. Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. Eur J Immunol. 1987; 17: 1297–1301. https://doi.org/10.1002/eji.1830170912 PMID: 2958295
4. Pescovitz MD, Lunney JK, Sachs DH. Murine anti-swine T4 and T8 monoclonal antibodies: distribution and effects on proliferative and cytotoxic T cells. J Immunol. 1985; 134: 37–44. PMID: 3871107
5. Blue ML, Daley JF, Levine H, Craig KA, Schlossman SF. Biosynthesis and surface expression of T8 by peripheral blood T4+ cells in vitro. J Immunol. 1986; 137: 1202–1207. PMID: 3090142
6. Akari H. Peripheral blood CD4+CD8+ lymphocytes in cynomolgus monkeys are of resting memory T lineage. International Immunology. 1997; 9: 591–597. https://doi.org/10.1093/intimm/9.4.591 PMID: 9138020
7. Takimoto H, Nakamura T, Takeuchi M, Sumi Y, Tanaka T, Nomoto K, et al. Age-associated increase in number of CD4+CD8+ intestinal intraepithelial lymphocytes in rats. Eur J Immunol. 1992; 22: 159–164. https://doi.org/10.1002/eji.1830220124 PMID: 1370412
8. Mosley RL, Styre D, Klein JR. CD4+ CD8+ murine intestinal intraepithelial lymphocytes. Int Immunol. 1990; 2: 361–365. https://doi.org/10.1093/intimm/2.4.361 PMID: 1980616
9. Luhtala M, Lassila O, Toivanen P, Vainio O. A novel peripheral CD4+CD8+ T cell population: Inheritance of CD8α expression on CD4+ T cells. Eur J Immunol. 1997; 27: 189–193. https://doi.org/10.1002/eji.1830270128 PMID: 9022017
10. Kato M, Watarai S, Nishikawa S, Iwasaki T, Kodama H. A Novel Culture Method of Canine Peripheral Blood Lymphocytes with Concanavalin A and Recombinant Human Interleukin-2 for Adoptive Immunotherapy. J Vet Med Sci. 2007; 69: 481–486. https://doi.org/10.1292/jvms.69.481 PMID: 17551220
11. Hoshino Y, Takagi S, Osaki T, Okumura M, Fujinaga T. Phenotypic Analysis and Effects of Sequential Administration of Activated Canine Lymphocytes on Healthy Beagles. J Vet Med Sci. 2008; 70: 581–588. https://doi.org/10.1292/jvms.70.581 PMID: 18628598
12. Schütze N, Raue R, Büttner M, Alber G. Inactivated parapoxvirus ovis activates canine blood phagocytes and T lymphocytes. Vet Microbiol. 2009; 137: 260–267. https://doi.org/10.1016/j.vetmic.2009.01.035 PMID: 19251383
13. Otani I, Ohta K, Ishikawa A, Yamada T, Ishinazaka T, Ohtaki T, et al. Flow Cytometric Analysis of Canine Umbilical Cord Blood Lymphocytes. J Vet Med Sci. 2008; 70: 285–287. https://doi.org/10.1292/jvms.70.285 PMID: 18388429
14. Rothe K, Bismarck D, Büttner M, Alber G, Buttlar H von. Canine peripheral blood CD4(+)CD8(+) double-positive Tcell subpopulations exhibit distinct Tcell phenotypes and effector functions. Vet Immunol Immunopathol. 2017; 185: 48–56. https://doi.org/10.1016/j.vetimm.2017.01.005 PMID: 28242002
15. Bismarck D, Schütze N, Moore P, Büttner M, Alber G, Buttlar Hv. Canine CD4+CD8+ double positive T cells in peripheral blood have features of activated T cells. Vet Immunol Immunopathol. 2012; 149: 157–166. https://doi.org/10.1016/j.vetimm.2012.06.014 PMID: 22789871
16. Bismarck D, Moore PF, Alber G, Buttlar H von. Canine CD4(+)CD8(+) double-positive T cells can develop from CD4(+) and CD8(+) T cells. Vet Immunol Immunopathol. 2014; 162: 72–82. https://doi.org/10.1016/j.vetimm.2014.09.008 PMID: 25454082
17. Cheroutre H, Lambolez F. Doubting the TCR coreceptor function of CD8alphaalpha. Immunity. 2008; 28: 149–159. https://doi.org/10.1016/j.immuni.2008.01.005 PMID: 18275828
18. Chizzolini C, Parel Y, Luca C de, Tyndall A, Akesson A, Scheja A, et al. Systemic sclerosis Th2 cells inhibit collagen production by dermal fibroblasts via membrane-associated tumor necrosis factor alpha. Arthritis Rheum. 2003; 48: 2593–2604. https://doi.org/10.1002/art.11129 PMID: 13130479
19. Kitchen SG, Jones NR, LaForge S, Whitmire JK, Vu B-A, Galic Z, et al. CD4 on CD8(+) T cells directly enhances effector function and is a target for HIV infection. Proc Natl Acad Sci U S A. 2004; 101: 8727–8732. https://doi.org/10.1073/pnas.0401500101 PMID: 15173593

20. Sarrabayrouse G, Bossard C, Chauvin J-M, Jarry A, Meurette G, Quévrain E, et al. CD4CD8αα lymphocytes, a novel human regulatory T cell subset induced by colonic bacteria and deficient in patients with inflammatory bowel disease. PLoS Biol. 2014; 12: e1001833. https://doi.org/10.1371/journal.pbio.1001833 PMID: 24714093

21. Bang K, Lund M, Wu K, Mogensen SC, Thestrup-Pedersen K. CD4+ CD8+ (thymocyte-like) T lymphocytes present in blood and skin from patients with atopic dermatitis suggest immune dysregulation. Br J Dermatol. 2001; 144: 1140–1147. https://doi.org/10.1046/j.1365-2133.2001.04223.x PMID: 11422033

22. Desfrançois J, Derré L, Corvaisier M, Le Mével B, Catros V, Jotereau F, et al. Increased frequency of nonconventional double positive CD4CD8 alphabeta T cells in human breast pleural effusions. Int J Cancer. 2009; 125: 374–380. https://doi.org/10.1002/ijc.24366 PMID: 19358272

23. McGill JL, Wang Y, Ganta CK, Boorgula GDY, Ganta RR. Antigen-Specific CD4+CD8+ Double-Positive T Cells Are Increased in the Blood and Spleen During Ehrlichia chaffeensis Infection in the Canine Host. Front Immunol. 2018; 9: 1585. https://doi.org/10.3389/fimmu.2018.01585 PMID: 30050533

24. Alexandre-Pires G, Brito MTV de, Algueró C, Martins C, Rodrigues OR, da Fonseca IP, et al. Canine leishmaniasis. Immunophenotypic profile of leukocytes in different compartments of symptomatic, asymptomatic and treated dogs. Vet Immunol Immunopathol. 2010; 137: 275–283. https://doi.org/10.1016/j.vetimm.2010.06.007 PMID: 20615553

25. Solano-Aguilar GI, Vengroski KG, Beshah E, Lunney JK. Isolation and purification of lymphocyte subsets from gut-associated lymphoid tissue in neonatal swine. J Immunol Methods. 2000; 241: 185–199. https://doi.org/10.1016/S0022-1759(00)00209-X PMID: 10915860

26. Mizuno T, Suzuki R, Umeki S, Okuda M. Crossreactivity of Antibodies to Canine CD25 and Foxp3 and Identification of Canine CD4+CD25 +Foxp3+ Cells in Canine Peripheral Blood. J Vet Med Sci. 2009; 71: 1561–1568. https://doi.org/10.1292/jvms.001561 PMID: 20046022

27. Junginger J, Schwittlick U, Lemensieck F, Nolte I, Hewicker-Trautwein M. Immunohistochemical investigation of Foxp3 expression in the intestine in healthy and diseased dogs. Vet Res. 2012; 43: 23. https://doi.org/10.1186/1297-9716-43-23 PMID: 22440243

28. Olivares-Villagómez D, van Kaer L. Intestinal Intraepithelial Lymphocytes: Sentinels of the Mucosal Barrier. Trends Immunol. 2018; 39: 264–275. https://doi.org/10.1016/j.it.2017.11.003 PMID: 29221933

29. Luckschander N, Pfammatter NS, Sidler D, Jakob S, Burgener IA, Moore PF, et al. Phenotyping, functional characterization, and developmental changes in canine intestinal intraepithelial lymphocytes. Vet Res. 2009; 40: 58. https://doi.org/10.1051/vetres/2009042 PMID: 19631032

30. Godfrey DI, Zlotnik A. Control points in early T-cell development. Immunol Today. 1993; 14: 547–553. https://doi.org/10.1016/0167-5699(93)90186-O PMID: 7903854

31. He YW, Nakajima H, Leonard WJ, Adkins B, Malek TR. The common gamma-chain of cytokine receptors regulates intrathymic T cell development at multiple stages. J Immunol. 1997; 158: 2592–2599. PMID: 9058791

32. Sinkora M, Sinkora J, Rehakova Z, Butler JE. Early Ontogeny of Thymocytes in Pigs: Sequential Colonization of the Thymus by T Cell Progenitors. J Immunol. 2000; 165: 1832–1839. https://doi.org/10.4049/jimmunol.165.4.1832 PMID: 10925261

33. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003; 4: 330–336. https://doi.org/10.1038/ni904 PMID: 12612578

34. Roederer M. How many events is enough? Are you positive. Cytometry A. 2008; 73: 384–385. https://doi.org/10.1002/cyto.a.20549 PMID: 18307257

35. Mayassi T, Jabri B. Human intraepithelial lymphocytes. Mucosal Immunol. 2018; 11: 1281–1289. https://doi.org/10.1038/s41385-018-0016-5 PMID: 29674648

36. Mucida D, Husain MM, Muroi S, van Wijk F, Shinnakasu R, Naoe Y, et al. Transcriptional reprogramming of mature CD4+ helper T cells generates distinct MHC class II-restricted cytotoxic T lymphocytes. Nat Immunol. 2013; 14: 281–289. https://doi.org/10.1038/ni.2523 PMID: 23334788

37. Takeuchi A, Saito T. CD4 CTL, a Cytotoxic Subset of CD4+ T Cells, Their Differentiation and Function. Front Immunol. 2017; 8: 194. https://doi.org/10.3389/fimmu.2017.00194 PMID: 28280496

38. Rütgen BC, König R, Hammer SE, Groiss S, Saalmüller A, Schwendenwein I. Composition of lymphocyte subpopulations in normal canine lymph nodes. Vet Clin Pathol. 2015; 44: 58–69. https://doi.org/10.1111/vcp.12221 PMID: 25512102

39. Sonea IM, Jergens AE, Sacco RE, Niyo Y, Merten E, Kauffman LK, et al. Flow cytometric analysis of colonic and small intestinal mucosal lymphocytes obtained by endoscopic biopsy in the healthy dog. Vet Immunol Immunopathol. 2000; 77: 103–119. PMID: 11068069

40. Kern P, Hussey RE, Spoerl R, Reinherz EL, Chang H-C. Expression, Purification, and Functional Analysis of Murine Ectodomain Fragments of CD8αα and CD8αβ Dimers. J Biol Chem. 1999; 274: 27237–27243. https://doi.org/10.1074/jbc.274.38.27237 PMID: 10480942

41. McNicol A-M, Bendle G, Holler A, Matjeka T, Dalton E, Rettig L, et al. CD8α/α homodimers fail to function as co-receptor for a CD8-dependent TCR. Eur J Immunol. 2007; 37: 1634–1641. https://doi.org/10.1002/eji.200636900 PMID: 17506031

42. Cawthon AG, Lu H, Alexander-Miller MA. Peptide Requirement for CTL Activation Reflects the Sensitivity to CD3 Engagement: Correlation with CD8αβ Versus CD8αα Expression. J Immunol. 2001; 167: 2577–2584. https://doi.org/10.4049/jimmunol.167.5.2577 PMID: 11509598

43. Spolski R, Li P, Leonard WJ. Biology and regulation of IL-2: from molecular mechanisms to human therapy. Nat Rev Immunol. 2018; 18: 648–659. https://doi.org/10.1038/s41577-018-0046-y PMID: 30089912

44. Lefrancois L. Phenotypic complexity of intraepithelial lymphocytes of the small intestine. J Immunol. 1991; 147: 1746–1751. PMID: 1716278

45. Cheroutre H. Starting at the beginning: new perspectives on the biology of mucosal T cells. Annu Rev Immunol. 2004; 22: 217–246. https://doi.org/10.1146/annurev.immunol.22.012703.104522 PMID: 15032579

46. Morrissey PJ, Charrier K, Horovitz DA, Fletcher FA, Watson JD. Analysis of the intra-epithelial lymphocyte compartment in SCID mice that received co-isogenic CD4+ T cells. Evidence that mature post-thymic CD4+ T cells can be induced to express CD8 alpha in vivo. J Immunol. 1995; 154: 2678–2686. PMID: 7876540

47. Aranda R, Sydora BC, McAllister PL, Binder SW, Yang HY, Targan SR, et al. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. J Immunol. 1997; 158: 3464–3473. PMID: 9120308

48. Reis BS, Rogoz A, Costa-Pinto FA, Taniuchi I, Mucida D. Mutual expression of the transcription factors Runx3 and ThPOK regulates intestinal CD4(+) T cell immunity. Nat Immunol. 2013; 14: 271–280. https://doi.org/10.1038/ni.2518 PMID: 23334789

49. Oliveira LdM, Teixeira FME, Sato MN. Impact of Retinoic Acid on Immune Cells and Inflammatory Diseases. Mediators Inflamm. 2018; 2018: 3067126. https://doi.org/10.1155/2018/3067126 PMID: 30158832

50. Reboldi A, Cyster JG. Peyer's patches: organizing B-cell responses at the intestinal frontier. Immunol Rev. 2016; 271: 230–245. https://doi.org/10.1111/imr.12400 PMID: 27088918

51. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song S-Y. Retinoic acid imprints gut-homing specificity on T cells. Immunity. 2004; 21: 527–538. https://doi.org/10.1016/j.immuni.2004.08.011 PMID: 15485630

52. Zuckermann FA, Gaskins HR. Distribution of porcine CD4/CD8 double-positive T lymphocytes in mucosa-associated lymphoid tissues. Immunology. 1996; 87: 493–499. https://doi.org/10.1046/j.1365-2567.1996.494570.x PMID: 8778039

53. Das G, Augustine MM, Das J, Bottomly K, Ray P, Ray A. An important regulatory role for CD4+CD8 alpha alpha T cells in the intestinal epithelial layer in the prevention of inflammatory bowel disease. Proc Natl Acad Sci U S A. 2003; 100: 5324–5329. https://doi.org/10.1073/pnas.0831037100 PMID: 12695566

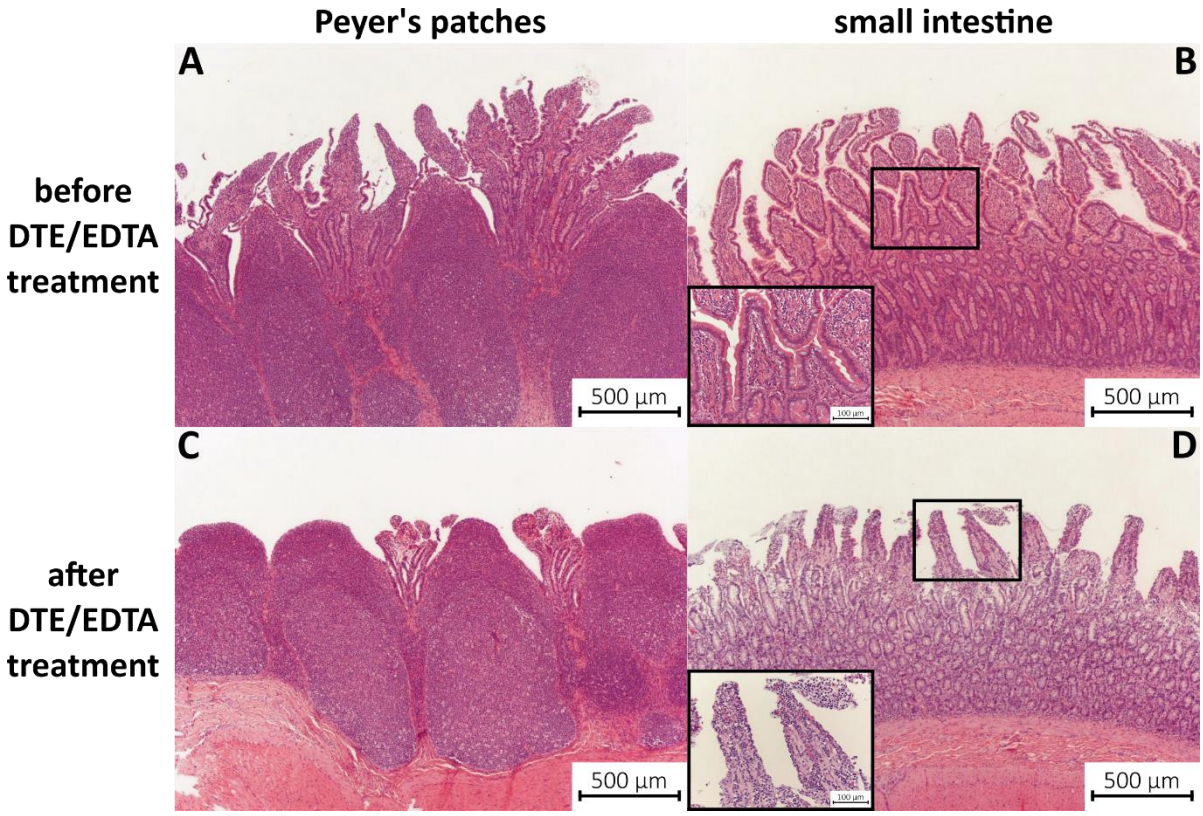
54. Hillemeyer P, White MD, Pascual DW. Development of a transient CD4+CD8+ T cell subset in the cervical lymph nodes following intratracheal instillation with an adenovirus vector. Cell Immunol. 2002; 215: 173–185. https://doi.org/10.1016/S0008-8749(02)00024-2 PMID: 12202154

55. Abuzakouk M, Carton J, Feighery C, O'Donoghue DP, Weir DG, O'Farrelly C. CD4+CD8+ and CD8α+β- T lymphocytes in human small intestinal lamina propria. Eur J Gastroenterol Hepatol. 1998; 10: 325–330. https://doi.org/10.1097/00042737-199804000-00009 PMID: 9855049

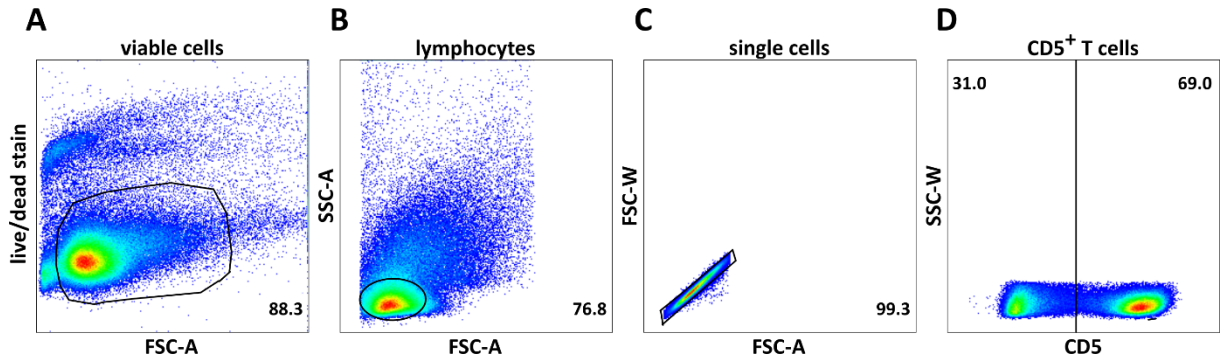
56. Käser T, Gerner W, Hammer SE, Patzl M, Saalmüller A. Detection of Foxp3 protein expression in porcine T lymphocytes. Vet Immunol Immunopathol. 2008; 125: 92–101. https://doi.org/10.1016/j.vetimm.2008.05.007 PMID: 18565594

57. van Kaer L, Rabacal WAS, Scott Algood HM, Parekh VV, Olivares-Villagómez D. In vitro induction of regulatory CD4+CD8α+ T cells by TGF-β, IL-7 and IFN-γ. PLoS ONE. 2013; 8: e67821. https://doi.org/10.1371/journal.pone.0067821 PMID: 23844100

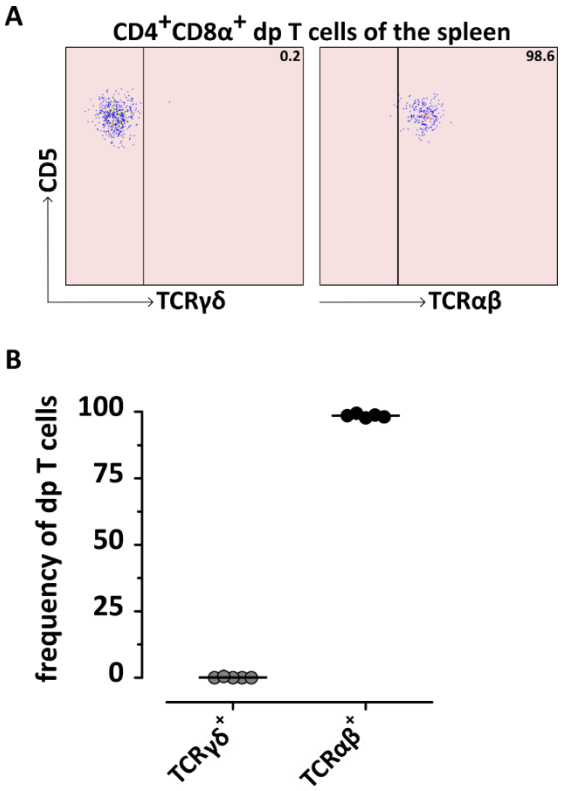
58. Sujino T, London M, van Hoytema Konijnenburg DP, Rendon T, Buch T, Silva HM, et al. Tissue adaptation of regulatory and intraepithelial CD4+ T cells controls gut inflammation. Science. 2016; 352: 1581–1586. https://doi.org/10.1126/science.aaf3892 PMID: 27256884



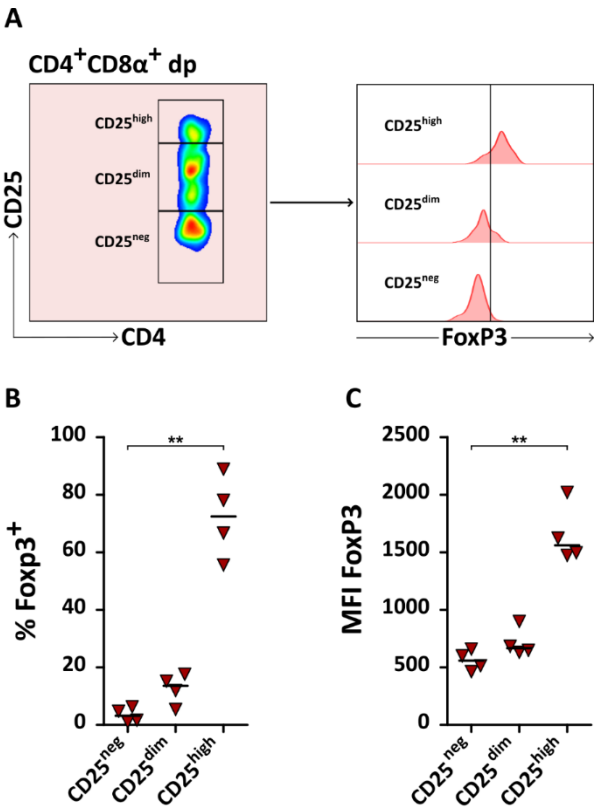
S1 Fig. The separation of the epithelium from Peyer's Patches and small intestinal villi was confirmed by hematoxylin and eosin (H&E) staining before and after treatment with DTE/EDTA. Representative H&E-stained sections of Peyer's patches (A) and small intestine (B) before DTE/EDTA treatment are shown. Normal villous architecture with intact epithelium is visible. After DTE/EDTA treatment, the epithelial layer is removed from Peyer's patches (C), and from the villi (D), respectively. For the latter this is displayed at higher magnifications in the insets (compare B + D).



S2 Fig. General gating strategy used in flow cytometric analyses. Representative pseudocolor plots of Peyer's patches are depicted to show the general gating strategy. After exclusion of dead cells (A), gating on lymphocytes was performed according to their forward and side scattering (FSC/SSC) properties (B). Following doublet-exclusion (C), only $CD5^+$ T cells were included into subsequent analyses (D).



S3 Fig. The absence of TCRγδ corresponds with the presence of TCRαβ on CD4$^+$CD8α$^+$ double-positive (dp) T cells. (A) The expression of TCRαβ and TCRγδ on splenic CD4$^+$CD8α$^+$ dp T cells was analyzed by flow cytometry. Shown are pseudocolor plots of one representative dog. (B) Proportions of TCRγδ (grey dots) vs. TCRαβ (black dots) expression of mature splenic CD4$^+$CD8α$^+$ dp T cells were quantified. Each dot represents one individual dog, the horizontal bars indicate mean values.



S4 Fig. In lymph nodes, FoxP3^{+}CD4^{+}CD8α^{+} double-positive (dp) T cells are mainly CD25^{high}. (A) Mesenteric lymph node CD4^{+}CD8α^{+} dp T cells were analyzed for CD25 and FoxP3 expression. Representative plots show the distribution of FoxP3^{+} cells in CD25^{neg}, CD25^{dim} and CD25^{high} subpopulations. The frequency (B) and mean fluorescence intensity (MFI) (C) of FoxP3 expression in CD25^{neg}, CD25^{dim}, and CD25^{high} CD4^{+}CD8α^{+} dp T cells in lymph nodes was quantified. Each symbol represents one individual dog, the horizontal bars indicate median values. Statistical analysis was performed by One-way ANOVA with Dunn's Multiple Comparison Test (** $p < 0.01$).

2.2 2nd publication

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Distinct features of canine non-conventional

$CD4^{-}CD8\alpha^{-}$ double-negative $TCR\alpha\beta^{+}$ vs. $TCR\gamma\delta^{+}$ T cells

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Gottfried Alber:

- conceptualization
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Distinct Features of Canine Non-conventional CD4$^-$CD8α$^-$ Double-Negative TCRαβ$^+$ vs. TCRγδ$^+$ T Cells

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The role of conventional TCRαβ$^+$CD4$^+$ or TCRαβ$^+$CD8α$^+$ single-positive (sp) T lymphocytes in adaptive immunity is well-recognized. However, non-conventional T cells expressing TCRαβ or TCRγδ but lacking CD4 and CD8α expression [i.e., CD4$^-$CD8α$^-$ double-negative (dn) T cells] are thought to play a role at the interface between the innate and adaptive immune system. Dn T cells are frequent in swine, cattle or sheep and predominantly express TCRγδ. In contrast, TCRγδ$^+$ T cells are rare in dogs. In this study, we identified a high proportion of canine dn T cells in the TCRαβ$^+$ T cell population of PBMC, lymphatic and non-lymphatic organs. In PBMC, the frequency of this T cell subpopulation made up one third of the frequency of TCRαβ$^+$CD4$^+$ sp, and almost half of the frequency of TCRαβ$^+$CD8α$^+$ sp T cells (i.e., ∼15% of all TCRαβ$^+$ T cells). Among TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells of PBMC and tissues, FoxP3$^+$ cells were identified indicating regulatory potential of this T cell subset. 80% of peripheral blood FoxP3$^+$TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells co-expressed CD25, and, interestingly, also the FoxP3-negative TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells comprised ∼34% CD25$^+$ cells. Some of the FoxP3-positive TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells co-expressed GATA-3 suggesting stable function of regulatory T cells. The frequency of GATA-3 expression by FoxP3$^-$TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells was even higher as compared with TCRαβ$^+$CD4$^+$ sp T cells (20.6% vs. 11.9%). Albeit lacking FoxP3 and CD25 expression, TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells also expressed substantial proportions of GATA-3. In addition, TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells produced IFN-γ and IL-17A upon stimulation. T-bet and granzyme B were only weakly expressed by both dn T cell subsets. In conclusion, this study identifies two dn T cell subsets in the dog: (i) a large (∼7.5% in Peyer's patches, ∼15% in lung) population of TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells with subpopulations thereof showing an activated phenotype, high expression of FoxP3 or GATA-3 as well as production of IFN-γ or IL-17A and (ii) a small TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cell subset also expressing GATA-3 without production of IFN-γ or IL-17A. It will be exciting to unravel the function of each subset during immune homeostasis and diseases of dogs.

Keywords: dog, canine T cells, CD4$^-$CD8α$^-$, double-negative, non-conventional T cells, TCRαβ, TCRγδ

INTRODUCTION

Dogs are important companion animals which develop a range of immune-mediated diseases such as allergies, cancer (e.g., mammary tumors), or autoimmune disorders that are very similar to those occurring in the human species (1, 2). With the dog living in close contact with people, it is not only worth studying these diseases for the dog itself, but it might also be a useful model to draw conclusions for humans. For this reason, it is essential to intensify research on the canine immune system which is still poorly understood.

Besides the well-known conventional single-positive (sp) T cells (i.e., CD4$^+$ and CD8α$^+$ sp T cells) there are extrathymic non-conventional CD4$^-$CD8α$^-$ double-negative (dn) T cells lacking the CD4 and CD8α co-receptors (3). These cells were described almost 30 years ago for man and mice (4, 5). Interestingly, even earlier "unusual subpopulations" of T cells missing expression of CD4 and CD8α were observed in swine (6, 7) and subsequently identified as TCRγδ$^+$ T cells (8, 9). In sheep, cattle, and chicken also early immunological studies unraveled the existence of high numbers of blood and tissue TCRγδ$^+$ T cells which were mostly CD4$^-$CD8α$^-$ dn T cells (10–14). With this pioneering research in veterinary immunology the concept of "γδ T cell high" species (e.g., swine, sheep, cattle, chicken) and "γδ T cell low" species (man, mouse) was established (15). Thus, comparative immunology has contributed to a broader and deeper view into the nature of non-conventional T cell populations, especially for γδ T cells. Studies looking at canine non-conventional lymphocyte subsets started later and characterized the dog as a "γδ T cell low" species (16). More recently, questions about the occurrence, regulation and function of CD4$^-$CD8α$^-$ dn T cells were raised in context with the investigation of regulatory T cell populations of healthy dogs (17), canine leishmaniasis (18), or upon specific immunotherapy for dogs with adverse food reactions (19).

For more extended functional aspects of CD4$^-$CD8α$^-$ dn T cells, of course, murine and human systems with ample reagents available proofed to be more accessible than research in domestic animals, albeit at the cost of a narrower scientific perspective. Thus, studies on CD4$^-$CD8α$^-$ dn T cell functions done in rodents and humans demonstrated immunoregulatory activity and a role in autoimmunity as reviewed recently (3, 20). Based on their regulatory potential, murine and human CD4$^-$CD8α$^-$ dn T cells have been termed "non-conventional regulators" (21).

Research on CD4$^-$CD8α$^-$ dn T cells of domestic animals nevertheless proceeded either driven by the generation of new monoclonal antibodies against species-specific markers relevant for research on CD4$^-$CD8α$^-$ dn T cells or by the identification of cross-reactive antibodies. Functional features of γδ T cells have been characterized in cattle where expression of WC1, a member of the CD163 family, was shown to act as a γδ T cell co-stimulatory receptor and pattern recognition receptor (PRR) for pathogenic bacteria (22, 23). In swine, a recent functional analysis of γδ T cells revealed distinct expression patterns of transcription factors and cytokines depending on the γδ T cell phenotypes (24). In dog, extrathymic non-conventional CD4$^-$CD8α$^-$ dn T cells (CD3$^+$, TCRαβ$^+$, or TCRγδ$^+$) have only been described in single studies (18, 19, 25) and a comprehensive characterization of these cells is still missing. Thus, we chose to perform a systematic multiparameter flow cytometry analysis of canine CD4$^-$CD8α$^-$ dn T cells. We found surprisingly high proportions of CD4$^-$CD8α$^-$ dn T cells in peripheral blood mononuclear cells (PBMC), lymphoid and non-lymphoid organs of healthy dogs. It is noteworthy that the majority of canine CD4$^-$CD8α$^-$ dn T cells is TCRαβ$^+$, with ∼ 1/3 expressing the activation marker CD25 and a substantial part of those CD25$^+$ cells expressing FoxP3. Subpopulations of these TCRαβ$^+$CD4$^-$CD8α$^-$ dn cells also express GATA-3 and produce almost comparable amounts of IFN-γ or IL-17A as their CD4$^+$ sp counterparts. On the other hand, they express only low frequencies of T-bet and granzyme B. In contrast, the small TCRγδ$^+$ CD4$^-$CD8α$^-$ dn T cell population does neither express markers of activation nor FoxP3, IFN-γ or IL-17A, but resembles its TCRαβ$^+$ counterpart regarding the high frequencies of GATA-3, and low frequencies of T-bet and granzyme B expressing cells.

By comparing immune cell subpopulations across different species, we gain a broader and deeper view into the nature and function of these cells, which ultimately will lead to the identification of the most suitable animal species serving as model for human diseases.

MATERIALS AND METHODS

Animals, Blood, and Tissue Samples

Venous blood was taken from 10 healthy experimental Beagle dogs (five female, five male, age: 3–9 years) into heparinized vacutainer tubes (BD Vacutainer®, 10 ml, Li-Heparin 17 IU/ml, Becton Dickinson, Heidelberg, Germany). At the time of blood sampling, the dogs belonged to the College of Veterinary Medicine, University of Leipzig, Germany. The Animal Care and Usage Committee of the Saxony State Office (*Landesdirektion Sachsen*) in Leipzig, Germany, authorized the study (approval numbers: A 10/14 and A 28/18).

Tissue samples were collected from another group of experimental Beagle dogs (Marshall Bioresources, North Rose, NY, USA, $n = 12$, six female, six male, age: 10–15 months). The dogs were clinically healthy animals which were euthanized for reasons unrelated to our studies (control group of an animal experiment for preclinical drug development, approval number V54-19c 20/15-DA4/Anz.1004). Necropsies and histopathological examinations confirmed the physical health of every single dog. Following euthanasia, full thickness sections from mesenteric (mLN) and tracheobronchial (tLN) lymph nodes, spleen, duodenum, jejunum, and lung were collected immediately for further processing (mLN, spleen, duodenal/jejunal Peyer's patches, lung: $n = 10$, tLN: $n = 9$).

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Whole blood was diluted in phosphate buffered saline (PBS) at a ratio of 1:1, layered above Biocoll Separating Solution (Biochrom AG, Berlin, Germany) and centrifuged at 500 × g for 30 min at room temperature (RT). After washing with PBS, cells were treated with erythrocyte lysis buffer (150 mM NH_4Cl,

8 mM $KHCO_3$, 2 mM EDTA; pH 7) for 5 min at RT and the lysis reaction was stopped with PBS containing 3% fetal bovine serum (FBS, Thermo Fisher Scientific, Carlsbad, USA; and PAN-Biotech, Aidenbach, Germany). Next, PBMC were washed with PBS and counted with a microscope using a hemocytometer (Laboroptik, Lancing, UK) and trypan blue (Sigma-Aldrich, Taufkirchen, Germany).

Stimulation of PBMC

PBMC were resuspended in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 100 U/ml penicillin, 100 µg/ml streptomycin (both purchased from PAA Laboratories), and 10% FBS (Thermo Fisher Scientific, Carlsbad, USA). Cells were cultured overnight (37°C, 5% CO_2) at a density of 5×10^5 cells per well in 96 well flat bottom plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland). Stimulation was done the next day with 0.22 µg/ml phorbol-myristate-acetate (PMA)/ionomycin for 4 h in combination with 5 µg/ml Brefeldin A. Medium incubation served as negative control.

Generation of Single Cell Suspensions of Lymph Nodes and Spleen

Leukocytes from mLN, tLN, and spleen were isolated as previously described (26). In brief, tissue pieces were minced, passed through a 100 µm nylon cell strainer (BD Biosciences, Heidelberg, Germany) and resuspended in PBS followed by lysis of erythrocytes and cell counting as mentioned earlier.

Isolation of Lymphocytes From Peyer's Patches

After collection from duodenum and jejunum, Peyer's Patches (PP) were immediately washed in ice-cold Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (PAN-Biotech, Aidenbach, Germany) supplemented with 10 mM HEPES (Carl Roth, Karlsruhe, Germany) and 2% FBS (Thermo Fisher Scientific, Carlsbad, USA; and PAN-Biotech, Aidenbach, Germany). Afterwards, the pieces were incubated 3 × 30 min in HBSS containing 2 mM 1,4-Dithioerythritol (DTE, Sigma-Aldrich, Taufkirchen, Germany), and 0.5 mM EDTA (Carl Roth, Karlsruhe, Germany) at 37°C under continuous stirring to remove the intestinal epithelium. The remaining tissue was minced, dissociated by the gentleMACS™ Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), and passed through a 100 µm cell strainer (BD Biosciences, Heidelberg, Germany). To purify lymphocytes, cells were centrifuged on a discontinuous density gradient with 40% and 70% Percoll (900 × g, 20 min, RT), harvested at the interphase, and washed in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 5% FBS and 50 µg/ml Gentamicin (Sigma-Aldrich, Taufkirchen, Germany; and PAN-Biotech, Aidenbach, Germany).

Isolation of Lung Leukocytes

Lung tissue was cut into small pieces and digested for 30 min at 37°C in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with DNase I (111 U/ml; Sigma-Aldrich, Taufkirchen, Germany), Collagenase D (0.7 mg/ml; Roche Diagnostics Deutschland GmbH, Mannheim, Germany), and 1 mM sodium pyruvate (AppliChem, Darmstadt, Germany). Following passage through 100 µm cell strainers (BD Biosciences, Heidelberg, Germany), erythrocytes were lysed as described above. Leukocytes were separated from tissue cells by 30%/70% Percoll (GE Healthcare, Uppsala, Sweden) gradient centrifugation (400 × g, 20 min, RT). Cells were recovered from the interphase and resuspended in IMDM medium (PAN-Biotech, Aidenbach, Germany) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (both purchased from PAA Laboratories), and 10% FBS (Thermo Fisher Scientific, Carlsbad, USA; and PAN-Biotech, Aidenbach, Germany).

Flow Cytometric Analysis

Fixable viability dye eFluor 780 (Thermo Fisher Scientific, Carlsbad, USA) was used according to the manufacturer's protocol to discriminate dead from viable cells. In a second step, they were incubated with a mixture of heat-inactivated normal serum derived from dog, rat, and mouse (each 15% in PBS) to block non-idiotypic binding. Next, surface staining was performed by incubating cells with primary antibodies for 15 min in the dark on ice. To detect canine TCRαβ and TCRγδ, a PerCP/Cy5.5-conjugated goat-anti-mouse IgG secondary antibody (Biolegend, San Diego, USA) was used. In this case, blockade of non-idiotypic binding was performed with a mixture of heat-inactivated rat, dog and goat normal serum (each 15% in PBS). If CD25 was included in the surface staining panel, incubation with the P4A10 antibody derived from mice was performed separately after an additional blocking step including mouse serum to saturate possible free binding sites of the goat-anti-mouse secondary antibody. The details of all primary antibodies used for flow cytometric staining are summarized in **Table 1**. Cross-reactivity of antibodies directed against non-canine antigens was validated by several groups (see references in **Table 1**) or reported by the supplier (e.g., anti-CD3ε clone CD3-12). For the anti-human/mouse GATA-3 antibody TWAJ, cross-reactivity with canine GATA-3 is very likely based on *in silico* epitope prediction by Kolaskar and Tongaonkar 1990 which leads to nearly identical epitopes predicted in the murine, canine and human sequence (37). Moreover, the homology of canine to murine GATA-3 is very high (above 95%).

If only surface staining was performed, the cells were fixed with 2% paraformaldehyde (Sigma-Aldrich, Taufkirchen, Germany) for 15 min in the dark on ice. For intracellular staining of CD3, cells were permeabilized with 0.5% saponine (Carl Roth, Karlsruhe, Germany) for 10 min at RT. Transcription factor staining and staining of granzyme B, IFN-γ, and IL-17A was performed using the FoxP3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Carlsbad, USA) according to the manufacturer's protocol. After permeabilization, an additional blocking step with dog, rat, and mouse normal serum was done and cells were incubated with antibodies for 30 min at RT. Following acquisition with a BD LSR Fortessa™ flow cytometer (Becton Dickinson, Heidelberg, Germany) cell samples were analyzed using the FlowJo™10 software (Treestar Inc., Ashland, OR, USA). For all flow cytometry plots, biexponential scaling

TABLE 1 | Primary antibodies used for flow cytometry.

Antigen	Clone	Species reactivity	Isotype	Source	Fluorochrome	References
TCRαβ	CA15.8G7	canine	Mouse IgG1	Leukocyte Antigen Biology Laboratory, Davis, USA	Hybridoma supernatant	not applicable (N/A)
TCRγδ	CA20.8H1	canine	Mouse IgG2a	Leukocyte Antigen Biology Laboratory, Davis, USA	Hybridoma supernatant	N/A
CD4	YKIX302.9	canine	Rat IgG2a	Thermo Fisher Scientific, Carlsbad, USA Bio-Rad, Munich, Germany	APC Pacific Blue, RPE	N/A
CD8α	YCATE55.9	canine	Rat IgG1	Thermo Fisher Scientific, Carlsbad, USA Bio-Rad, Munich, Germany	APC, PerCP-eFluor 710 Pacific Blue, Alexa Fluor 647	N/A
CD25	P4A10	canine	Mouse IgG1	Thermo Fisher Scientific, Carlsbad, USA	PE	N/A
FoxP3	FJK-16s	mouse/rat	Rat IgG2a	Thermo Fisher Scientific, Carlsbad, USA	FITC	(26–29)
GATA-3	TWAJ	human/mouse	Rat IgG2b	Thermo Fisher Scientific, Carlsbad, USA	eFluor 660	–
T-bet	eBio4B10	human/mouse	Mouse IgG1	Thermo Fisher Scientific, Carlsbad, USA	eFluor 660	(27, 30)
IFN-γ	CC302	bovine	Mouse IgG1	Bio-Rad, Munich, Germany	RPE	(27, 31–33)
IL-17A	eBio64DEC17	human	Mouse IgG1	Thermo Fisher Scientific, Carlsbad, USA	Alexa Fluor 488	(34, 35)
Granzyme B	GB11	human/mouse	Mouse IgG1	Biolegend, San Diago, USA	FITC	(26, 27)
CD5	YKIX322.3	canine	Rat IgG2a	Thermo Fisher Scientific, Carlsbad, USA	PE, PerCP-eFluor 710	N/A
CD3	CD3-12	human	Rat IgG1	Bio-Rad, Munich, Germany	FITC	(36)

was used which can be retraced in **Supplemental Figures 1, 2**. Scales were not changed within figures. After exclusion of dead cells, lymphocytes were gated with respect to their size and granularity (**Figure 1**). Adequate gating was performed by including Fluorescence Minus One (FMO) controls in the experiments. Within FMO controls, the antibody of interest is replaced by its isotype control (all purchased from Thermo Fisher Scientific, Carslbad, USA; or Biolegend, San Diego, USA), whereas all other specific antibodies of the staining panel are included. As suggested by Roederer, for samples with a low number of events only signals with a comparable distribution of fluorescence as appropriate positive controls were assessed as positive (38).

Statistical Analysis

Statistical analysis of data was done using Graph Pad Prism 5.01 (San Diego, CA, USA) software. To test for normality, Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lillie for *p*-value) was applied. Normally distributed data sets are presented with the mean. For comparison of two normally distributed and independent groups, the unpaired Student's *t*-test (two-tailed) was used, whereas differences between more than two groups were analyzed by One-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test. Nonparametric data are shown with the median. In this case, multiple comparisons were performed by use of the Kruskal-Wallis H test with Dunn's post-test. Comparison of two independent groups was performed using the Mann-Whitney U test (two-tailed). The level of confidence for significance is shown in figure legends.

RESULTS

High Frequencies of CD4$^-$CD8α$^-$ Double-Negative T Cells Can Be Found Within Canine TCRαβ$^+$ T Cells of Peripheral Blood, Lymphatic, and Non-lymphatic Organs

CD4$^-$CD8α$^-$ double-negative (dn) T cells in canine species have been observed in former studies (18, 19, 25), but an in-depth characterization of these cells is still missing. Here we analyze the distribution of CD4$^-$CD8α$^-$ dn T cells within several lymphatic and non-lymphatic organs of the dog, i.e., peripheral blood mononuclear cells (PBMC), mesenteric (mLN) and tracheobronchial (tLN) lymph nodes, spleen, Peyer's patches (PP), and lung. Viable lymphocytes were gated on either TCRαβ$^+$ or TCRγδ$^+$ T cells (**Figures 1A–E**). Regarding the large TCRαβ$^+$ lymphocyte subset [mean 79.2% of all lymphocytes (PBMC), **Supplemental Figure 3**], we were surprised to find a substantial proportion (median values up to 15%) of TCRαβ$^+$ cells expressing neither CD4 nor CD8α, with highest frequencies in lung (median 15%), and lowest frequencies in Peyer's patches (median 7.5%, **Figures 1D,F,G**). Within PBMC, the frequency of TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells corresponds to about one third of the frequency of TCRαβ$^+$CD4$^+$ and about half of the frequency of TCRαβ$^+$CD8α$^+$ single-positive (sp) T cells (i.e., median 14.4% of all TCRαβ$^+$ T cells, **Supplemental Figures 4A,B**). This finding is in clear contrast to other species, like swine, humans, or mice, where TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells of peripheral blood only comprise a very small proportion (up to 5%) of all T cells (39–41).

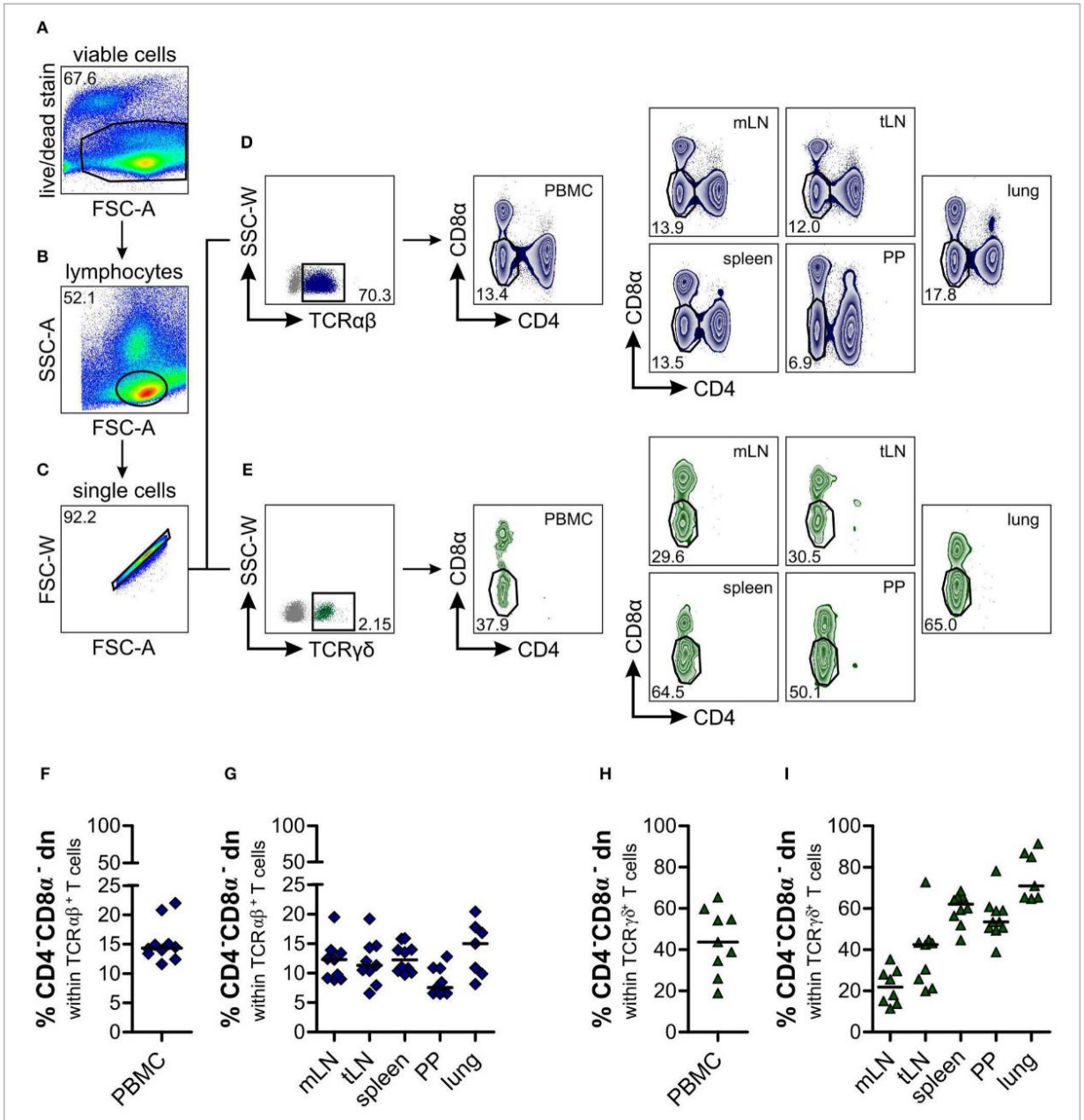

FIGURE 1 | Identification of a substantial proportion of TCRαβ⁺CD4⁻CD8α⁻ double-negative (dn) T cells in different organs of dogs. Leukocytes from lymphatic and non-lymphatic organs of healthy Beagle dogs ($n = 7–10$), i.e., peripheral blood mononuclear cells (PBMC), mesenteric lymph nodes (mLN), tracheobronchial lymph nodes (tLN), spleen, Peyer's patches (PP), and lung were analyzed by multicolor flow cytometry. Viable lymphocytes **(A,B)** were gated after doublet-exclusion **(C)** on TCRαβ⁺ (**D**, left panel) resp. TCRγδ⁺ (**E**, left panel) T cells. Representative zebra plots of TCRαβ⁺CD4⁻CD8α⁻ dn T cells are shown in dark blue (**D**, middle and right panels), green zebra plots show TCRγδ⁺CD4⁻CD8α⁻ dn T cells of organs indicated above (**E**, middle and right panels). Numbers in plots imply percentages. **(F–I)** Quantification of TCRαβ⁺CD4⁻CD8α⁻ (**F, G**; dark blue diamonds) and TCRγδ⁺CD4⁻CD8α⁻ dn T cells (**H, I**; green triangles) in indicated organs. Each symbol represents one individual dog, the horizontal bars display median values. Peripheral blood ($n = 9–10$) was taken from a different group of Beagle dogs than tissues ($n = 7–10$).

As expected, frequencies of TCRγδ$^+$ T cells were rather low in all analyzed organs (mean frequencies 0.5–4.6%; **Figure 1E**, **Supplemental Figure 3**) which is in line with data by Faldyna et al. for PBMC, spleen, and lymph nodes (16) and characterizing the dog as "γδ T cell low species." Within this small TCRγδ$^+$ cellular population, we did not observe CD4$^+$ sp or CD4$^+$CD8α$^+$ double-positive T cells, but CD8α$^+$ sp and about 20–80% CD4$^-$CD8α$^-$ dn T cells (**Figures 1E,H,I**, **Supplemental Figures 4C,D**).

Canine TCRαβ$^+$CD4$^-$CD8α$^-$ Double-Negative T Cells Show Features of Effector Cells

To determine the phenotype and differentiation status of canine TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells, we were interested in surface expression of the activation marker CD25. In contrast to human TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells (40, 42), a high proportion (mean 36.02%) of canine TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells of PBMC expresses CD25. Compared to TCRαβ$^+$CD4$^+$ and TCRαβ$^+$CD8α$^+$ sp T cells, frequencies of CD25 expression are significantly increased in the TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cell subpopulation (**Figures 2A,C**). Besides, only TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells express CD25, whereas TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells are CD25$^-$ (**Figures 2B,D**).

In addition, we wished to analyze expression of CD5 by canine CD4$^-$CD8α$^-$ dn T cells and to compare TCRαβ$^+$ with TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cell subsets. CD5 is primarily expressed on T cells and can be used as T cell marker (43). Moreover, similar to WC1 expressed on bovine γδ T cells, CD5 is composed of scavenger receptor cysteine-rich protein domains characteristic for members of the CD163 family (44, 45). Co-staining of CD3 with CD5 revealed that CD3$^+$ T cells of PBMC express CD5 either at a high (CD5^{high}) or at an intermediate level (CD5^{int}, **Figure 3A**). Interestingly, the mean fluorescence intensity (MFI) for CD5 of TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells is significantly decreased in comparison with TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells (**Figures 3C,D**) defining most TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells as a CD5^{int} subset. Of note, a very small proportion of TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells does not express CD5 (**Figure 3B**). In conclusion, CD5^{high}CD4$^-$CD8α$^-$ dn cells can be assumed to be TCRαβ$^+$. This conclusion was also confirmed by analyzing CD5^{high}CD4$^-$CD8α$^-$ dn vs. TCRαβ$^+$CD4$^-$CD8α$^-$ dn PBMC, as mean frequencies of CD25 expression by both T cell subpopulations were very similar (**Supplemental Figures 5A–D**).

Regarding CD25 expression of CD5^{high}CD4$^-$CD8α$^-$ dn T cells in tissues, we observed intermediate (lymph nodes) to high proportions (spleen, Peyer's patches, lung) positive for the activation marker CD25. Interestingly, highest frequencies (up to 60% on average) of CD25-positive CD5^{high}CD4$^-$CD8α$^-$ dn T cells could be found within lung tissue (**Supplemental Figures 5E–G**).

CD4$^-$CD8α$^-$ Double-Negative TCRαβ$^+$ and TCRγδ$^+$ T Cells Differ in Expression of FoxP3, IFN-γ, and IL-17A, but Share High GATA-3 Expression

As TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells of mice and humans are involved in modulating immune responses (40, 42, 46), and given the high frequencies of CD25 expression of canine TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells (see **Figure 2**), we were especially interested in FoxP3 expression of these cells. For TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells of PBMC, FoxP3 expression could be detected at comparable levels as for their TCRαβ$^+$CD4$^+$ single-positive counterparts (mean ∼6.7%, **Figures 4A,C**). On the contrary, TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells of PBMC are FoxP3-negative (**Figure 4B**).

As expected, the mean fluorescence intensity of CD25 within the FoxP3 expressing TCRαβ$^+$CD4$^-$CD8α$^-$ dn subset was higher than within the FoxP3$^-$ counterpart (**Figures 4D,E**). However, a substantial proportion (mean 34.4%) of the latter subset is CD25$^+$, corresponding to an effector phenotype (**Figure 4F**).

In addition, we observed FoxP3 expression of CD5^{high}CD4$^-$CD8α$^-$ dn T cells in tissues. Median frequencies vary between 1% in mesenteric lymph node and ∼3.2% in spleen (**Supplemental Figure 6**).

Next, we investigated whether CD4$^-$CD8α$^-$ dn T cells exhibit properties of T helper (Th) 2, Th1, Th17, or cytotoxic T cells by analyzing expression of the transcription factors GATA-3 and T-bet, of the cytokines IFN-γ and IL-17A as well as of the cytotoxicity marker granzyme B. For both αβ and γδ T cells, we observed only low proportions of GATA-3$^+$ cells within the CD8α$^+$ sp subset. On the other hand, TCRαβ$^+$CD4$^+$ sp and both (TCRαβ$^+$ and TCRγδ$^+$) CD4$^-$CD8α$^-$ dn T cell subsets express GATA-3. Interestingly, frequencies of GATA-3 expressing TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells were even higher (though not significantly elevated) as compared with conventional TCRαβ$^+$CD4$^+$ sp T cells (median 20.6% vs. 11.9%). For αβ T cells, a second GATA-3$^+$ subset could be identified in CD4$^+$ sp and CD4$^-$CD8α$^-$ dn T cells co-expressing the Treg transcription factor FoxP3 (**Figure 5**).

As shown previously for dogs, the transcription factor T-bet is constitutively expressed by canine CD8α$^+$ sp, but only at a very low degree by CD4$^+$ sp peripheral T cells (27). Similar to the latter, we observed significantly lower T-bet expression of both TCRαβ$^+$ and TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells of canine PBMC in comparison to CD8α$^+$ sp T cells (**Figure 6**). For CD5^{high}CD4$^-$CD8α$^-$ dn T cells of tissues, low T-bet expression in comparison to CD8α$^+$ sp T cells was found as well (**Supplemental Figure 7**).

To study cytokine production by CD4$^-$CD8α$^-$ dn T cells, we stimulated PBMC with PMA/ionomycin and looked for production of IFN-γ and IL-17A. IFN-γ was elevated in TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells as compared with the medium control sample. Moreover, significantly increased IFN-γ production was found for their CD4$^+$ and CD8α$^+$ sp counterparts (**Figures 7A,B**). For γδ T cells, IFN-γ could only

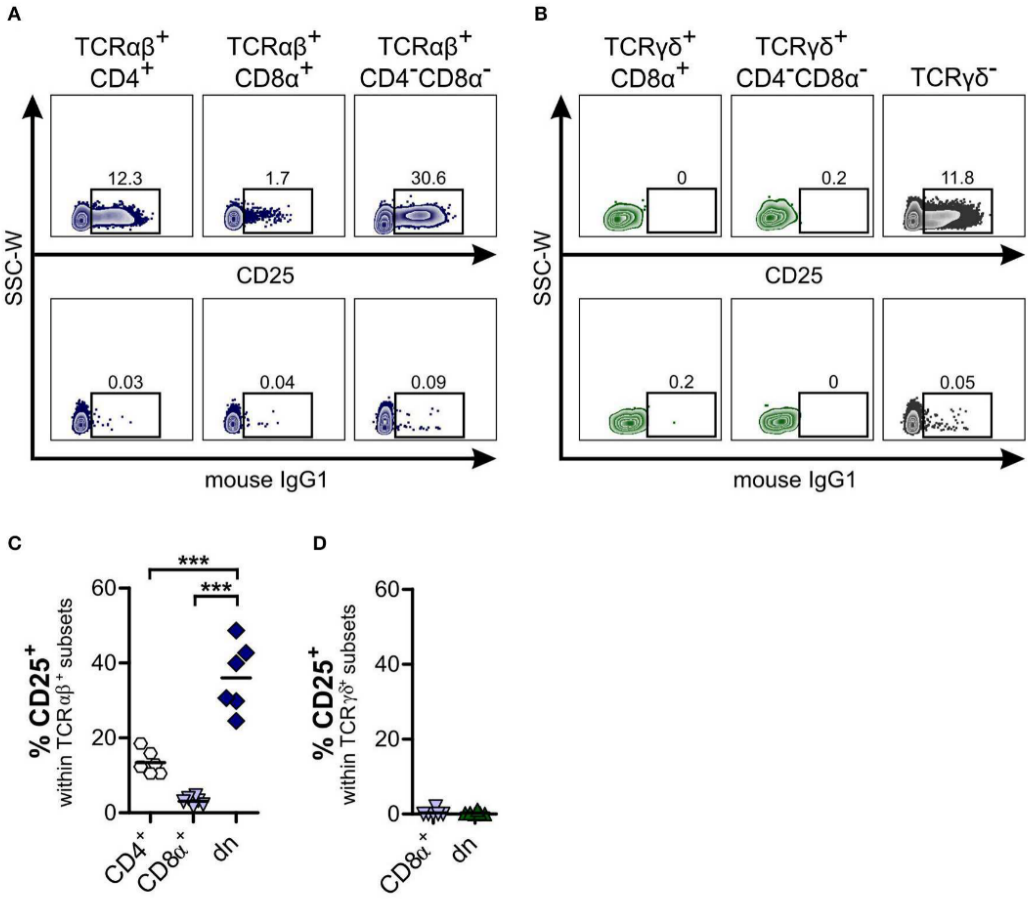

FIGURE 2 | About one third of canine TCRαβ⁺CD4⁻CD8α⁻ double-negative (dn) T cells is activated. Subpopulations of αβ and γδ T cells within PBMC were analyzed for CD25 expression. **(A,B)** CD25 staining of TCRαβ⁺ **(A)** resp. TCRγδ⁺ **(B)** T cell subsets of peripheral blood mononuclear cells (PBMC) in representative zebra plots (upper panels). Gating was performed according to Fluorescence Minus One (FMO) controls (lower panels). For γδ T cells, TCRγδ⁻ cells were analyzed for CD25 expression as staining control (**B**, right panel). **(C,D)** CD25 expression of αβ and γδ T cells was quantified. Shown are frequencies of TCRαβ⁺ ($n = 6$; CD4⁺ sp, white hexagons; CD8α⁺ sp, light blue triangles; CD4⁻CD8α⁻ dn, dark blue diamonds) **(C)**, and TCRγδ⁺ ($n = 6$; CD8α⁺ sp, light blue triangles; CD4⁻CD8α⁻ dn, green triangles) **(D)** subsets expressing CD25. For **(C,D)**, each symbol represents one single dog, the horizontal bars indicate mean values. Statistical analysis was performed by One-way analysis of variance (ANOVA) with Bonferroni's Multiple Comparison Test (***$p < 0.001$).

be observed within TCRγδ⁺CD8α⁺ sp T cells, whereas this cytokine was barely detectable within TCRγδ⁺CD4⁻CD8α⁻ dn T cells (**Figures 7A,C**). Interestingly, TCRαβ⁺CD4⁻CD8α⁻ dn T cell subsets produce the pro-inflammatory cytokine IL-17A upon PMA/ionomycin stimulation, similar to TCRαβ⁺CD4⁺ sp, and in contrast to TCRαβ⁺CD8α⁺ sp T cells (**Figures 7A,B**). Contrary to murine, human, porcine and bovine γδ T cells (47–51), canine γδ T cells (CD8α⁺ sp and CD4⁻CD8α⁻ dn) do not appear to express IFN-γ or IL-17A upon stimulation with PMA/ionomycin (**Figures 7A,C**).

Finally, we looked for expression of the cytotoxic molecule granzyme B within TCRαβ⁺ and TCRγδ⁺CD4⁻CD8α⁻ dn T cell subsets. For TCRαβ⁺CD4⁻CD8α⁻ dn PBMC, no granzyme B expression could be detected in the resting state (**Figures 8A,C**). Regarding γδ T cells, we observed granzyme B expression by both CD8α⁺ sp and CD4⁻CD8α⁻ dn T cells, even though granzyme B expression of CD4⁻CD8α⁻ dn T cells was significantly decreased in comparison with CD8α⁺ sp γδ T cells (**Figures 8B,D**). For tissues, too, only low frequencies of granzyme B were observed within the $CD5^{high}$CD4⁻CD8α⁻ dn fraction (**Supplemental Figure 8**).

Taken together, these data show that canine TCRαβ⁺CD4⁻CD8α⁻ dn T cells are a large population within all TCRαβ⁺ cells. They comprise surprisingly high numbers of effector T cells and subsets expressing FoxP3 and/or GATA-3, along with IFN-γ or IL-17A producing cells. On the other hand, the small subset of γδ T cells consists of CD8α⁺ sp and CD4⁻CD8α⁻ dn T cells with the latter expressing GATA-3

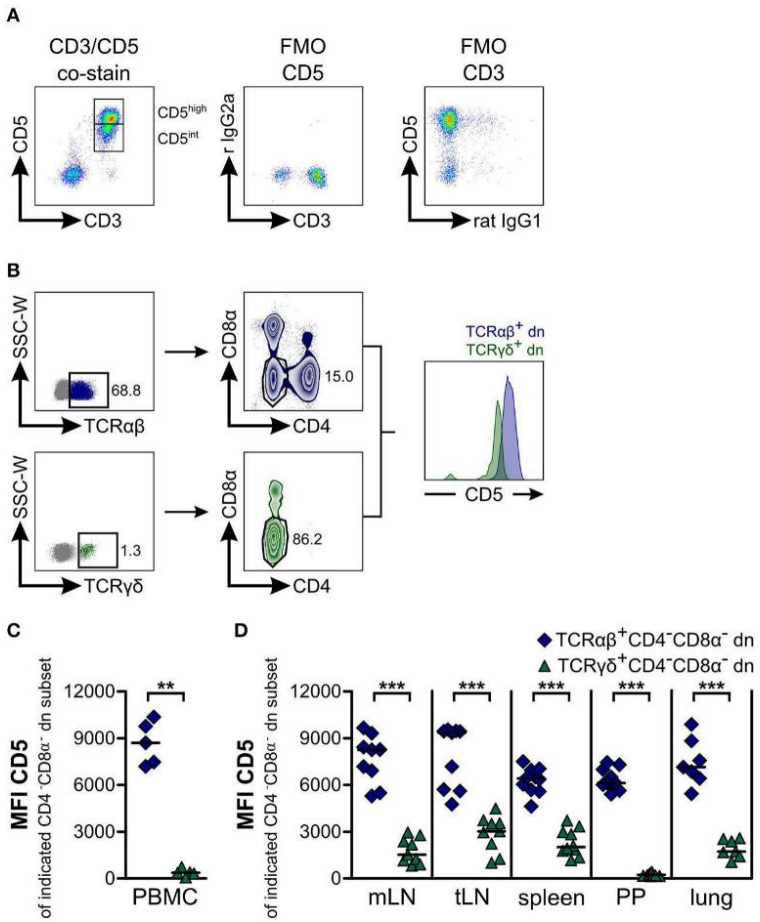

FIGURE 3 | Most TCRαβ$^+$CD4$^-$CD8α$^-$ double-negative (dn) T cells express high levels of CD5, whereas TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells are CD5^{intermediate}. **(A)** Canine peripheral blood mononuclear cells (PBMC) were stained for CD5 and CD3 expression. A representative pseudocolor plot of PBMC gated on living lymphocytes shows co-expression of CD3 and CD5 identifying the population as T cells. CD5$^+$ T cells can be divided into CD5^{high} and CD5^{intermediate} (CD5^{int}) subpopulations (left panel). Fluorescence Minus One (FMO) controls prove specific staining of antibodies (middle and right panel) (r IgG2a: rat IgG2a). **(B)** Representative plots of canine lung cells showing CD5 expression of TCRαβ$^+$CD4$^-$CD8α$^-$ (dark blue) and TCRγδ$^+$CD4$^-$CD8α$^-$ (green) dn T cells. **(C,D)** Mean Fluorescence Intensity (MFI) of CD5 expression by CD4$^-$CD8α$^-$ dn T cells pregated on TCRαβ (dark blue diamonds) or TCRγδ (green triangles) is shown. Each symbol represents one single dog, the horizontal bars indicate median values. Statistical analysis was performed by Mann-Whitney U test (two-tailed; $^{**}p < 0.01$, $^{***}p < 0.001$). Peripheral blood ($n = 5$) was taken from another group of Beagle dogs than tissues ($n = 7$–10).

as well as some T-bet and granzyme B but lacking IFN-γ and IL-17A production.

DISCUSSION

To date, canine extrathymic non-conventional CD4$^-$CD8α$^-$ double-negative (dn) T cells (CD3$^+$, TCRαβ$^+$, or TCRγδ$^+$) have only been observed in few previous studies where their potential importance was discussed (17–19, 25). To provide the basis for an in-depth understanding of these cells, we undertook a multiparameter flow cytometry analysis of canine TCRαβ$^+$ and TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells revealing their (i) frequency and tissue distribution, (ii) activation state, and (iii) functional potency by analysis of transcription factor (FoxP3, GATA-3, T-bet), cytokine (IFN-γ, IL-17A), and cytotoxicity marker (i.e., granzyme B) expression. The data obtained demonstrate a large subset of peripheral blood and tissue TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells with surprisingly high activation and an effector phenotype expressing FoxP3 and/or GATA-3. In contrast, canine TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells hardly express T-bet or granzyme B. With these features they clearly differ from their less numerous TCRγδ$^+$CD4$^-$CD8α$^-$ dn counterparts, and even more from conventional TCRαβ$^+$CD8α$^+$ sp T cells. On the other hand, we found phenotypic similarities to TCRαβ$^+$CD4$^+$ sp T cells, even though their activation state is remarkably

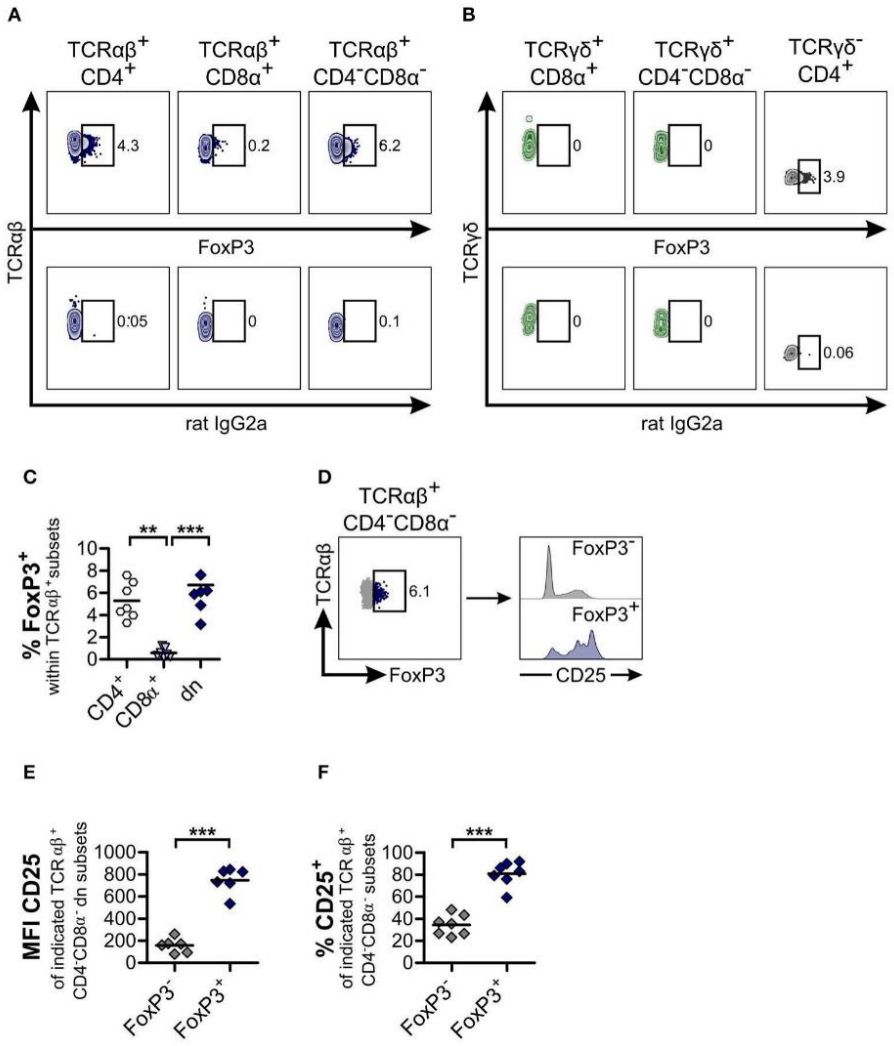

FIGURE 4 | TCRαβ⁺CD4⁻CD8α⁻ double-negative (dn) T cells contain as many FoxP3⁺ cells as TCRαβ⁺CD4⁺ single-positive (sp) T cells. **(A,B)** TCRαβ⁺ **(A)** resp. TCRγδ⁺ **(B)** T cell subsets of peripheral blood mononuclear cells (PBMC) were stained for FoxP3 expression. Shown are representative zebra plots of the corresponding T cell subsets (upper panels). Gating was performed according to Fluorescence Minus One (FMO) controls (lower panels). For γδ T cells, TCRγδ⁻CD4⁺ T cells were analyzed for FoxP3 expression as staining control (**B**, right panel). **(C)** Frequencies of FoxP3⁺ cells within TCRαβ⁺ subsets: CD4⁺ sp (white hexagons), CD8α⁺ sp (light blue triangles), and CD4⁻CD8α⁻ dn (dark blue diamonds) T cells from PBMC. Each symbol represents one individual dog, the horizontal bars indicate mean values ($n = 7$). Statistical analysis was performed by One-way analysis of variance (ANOVA) with Bonferroni's Multiple Comparison Test (**$p < 0.01$, ***$p < 0.001$). **(D)** Co-staining of CD25 and FoxP3 was performed. CD25 expression of TCRαβ⁺FoxP3⁻ (gray) and TCRαβ⁺FoxP3⁺ (blue) dn T cells is depicted. Shown are representative plots of one dog out of seven. **(E,F)** Mean Fluorescence Intensity (MFI) **(E)** and frequencies **(F)** of CD25 expression of FoxP3⁻ (gray diamonds) and FoxP3⁺ (blue diamonds) dn T cells are shown. For (F), gating on CD25⁺ cells was performed as shown in **Figure 2**. Each symbol represents one single dog, the horizontal bars indicate mean values ($n = 7$). Statistical analysis was performed by unpaired Student's *t*-test (two-tailed, ***$p < 0.001$).

different from the latter and rather comparable to canine TCRαβ⁺CD4⁺CD8α⁺ double-positive T cells (26, 27, 52).

A majority of canine CD4⁻CD8α⁻ dn T cells was found to express mainly TCRαβ, similar to murine and human CD4⁻CD8α⁻ dn T cells of lymphoid tissues. However, in the dog the proportion in peripheral blood or lymphoid tissues is clearly higher than in mouse or man (40, 41, 53). This of course raises the question related to the function of such a large non-conventional TCRαβ⁺ T cell subset in dogs, but also related to the function of the smaller TCRγδ⁺ cell subset with its remarkably high expression of GATA-3. Answers to these important questions will only be possible after additional CD4⁻CD8α⁻ dn T cell studies have been done in dogs during immune homeostasis or immune activation, e.g., in

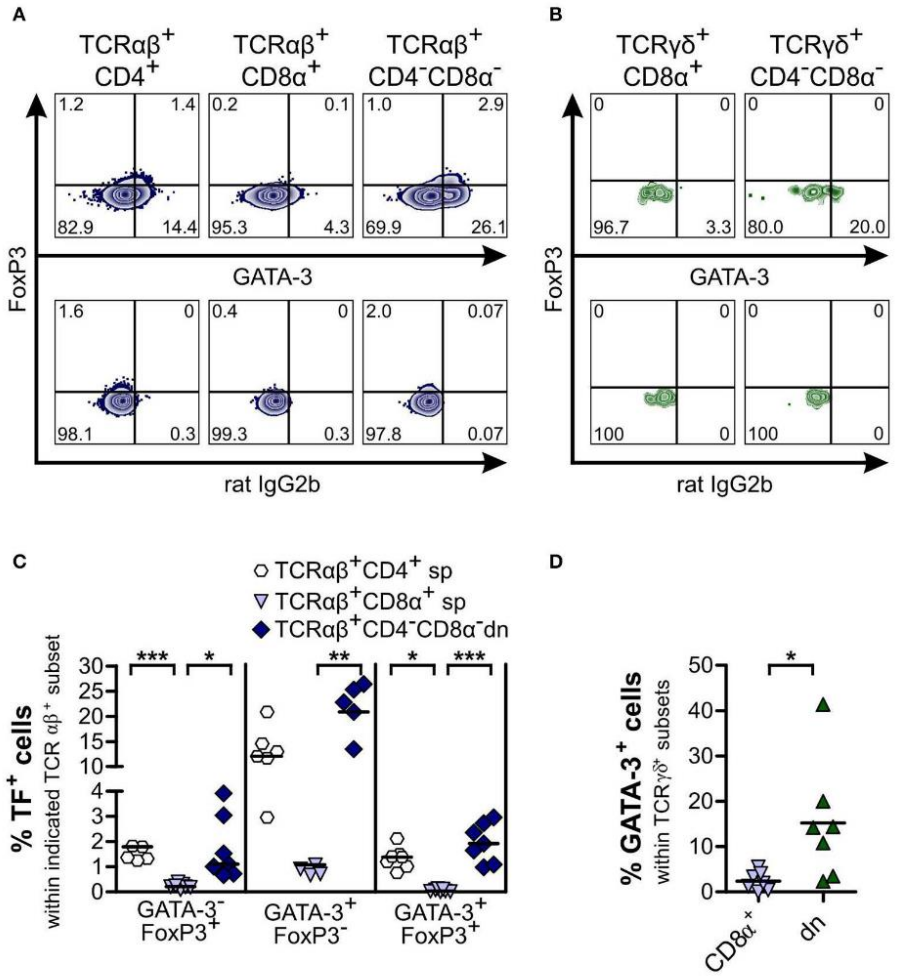

FIGURE 5 | Subsets of CD4⁻CD8α⁻ double-negative (dn) TCRαβ⁺ and TCRγδ⁻ T cells are GATA-3⁺, with a portion of TCRαβ⁺CD4⁻CD8α⁻ dn T cells consisting of GATA-3⁺FoxP3⁺ hybrid cells. Co-staining of FoxP3 and GATA-3 was performed. **(A,B)** Representative zebra plots of αβ **(A)** and γδ T cells **(B)** from PBMC analyzed for expression of FoxP3 and GATA-3 (upper panels). Appropriate gating was confirmed by Fluorescence Minus One controls (lower panels). Numbers in plots represent percentages. **(C)** Quantification of transcription factor (TF) (i.e., GATA-3 and FoxP3) expression of αβ T cell subsets: CD4⁺ sp (white hexagons), CD8α⁺ sp (light blue triangles), and CD4⁻CD8α⁻ dn (dark blue diamonds) T cells ($n = 7$). The horizontal bars indicate median values. Statistical analysis was performed by One-way analysis of variance (ANOVA) with Dunn's Multiple Comparison Test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(D)** GATA-3⁺ cells within TCRγδ⁺ subsets were quantified: CD8α⁺ sp (light blue triangles) and CD4⁻CD8α⁻ dn (green triangles) T cells ($n = 7$). The horizontal bars indicate mean values. Statistical analysis was performed by unpaired Student's *t*-test (two-tailed, *$p < 0.05$). For **(C,D)**, each symbol represents one individual dog.

context with immunization/infection, cancer, autoimmunity, or allergy.

It was surprising to find activation of a high portion of canine TCRαβ⁺CD4⁻CD8α⁻ dn T cells. Similar studies in human or murine TCRαβ⁺CD4⁻CD8α⁻ dn T cells show about 7–10-fold lower portions of CD25 expressing cells in peripheral blood or spleen (40, 54). Of note, within the murine urogenital tract elevated frequencies of activated TCRαβ⁺CD4⁻CD8α⁻ dn T cells have been described (41, 55). Currently the mechanisms driving high activation of canine TCRαβ⁺CD4⁻CD8α⁻ dn T cells in peripheral blood or lymphoid tissues remain elusive. TCRαβ-mediated triggering (leading to loss of naïve status and acquisition of an effector phenotype) and/or pattern recognition receptor (PRR)-dependent stimulation (e.g., at epithelial barriers) may contribute to activation of canine TCRαβ⁺CD4⁻CD8α⁻ dn T cells. The nature of the antigens responsible for TCRαβ-mediated activation and/or the type of PRR as well as the pathogen-associated molecular pattern(s) (PAMPs) for co-stimulation are currently unknown.

Expression of transcription factors has been demonstrated to determine subset-specific T cell function. To this end we analyzed expression of FoxP3, GATA-3, and T-bet by canine

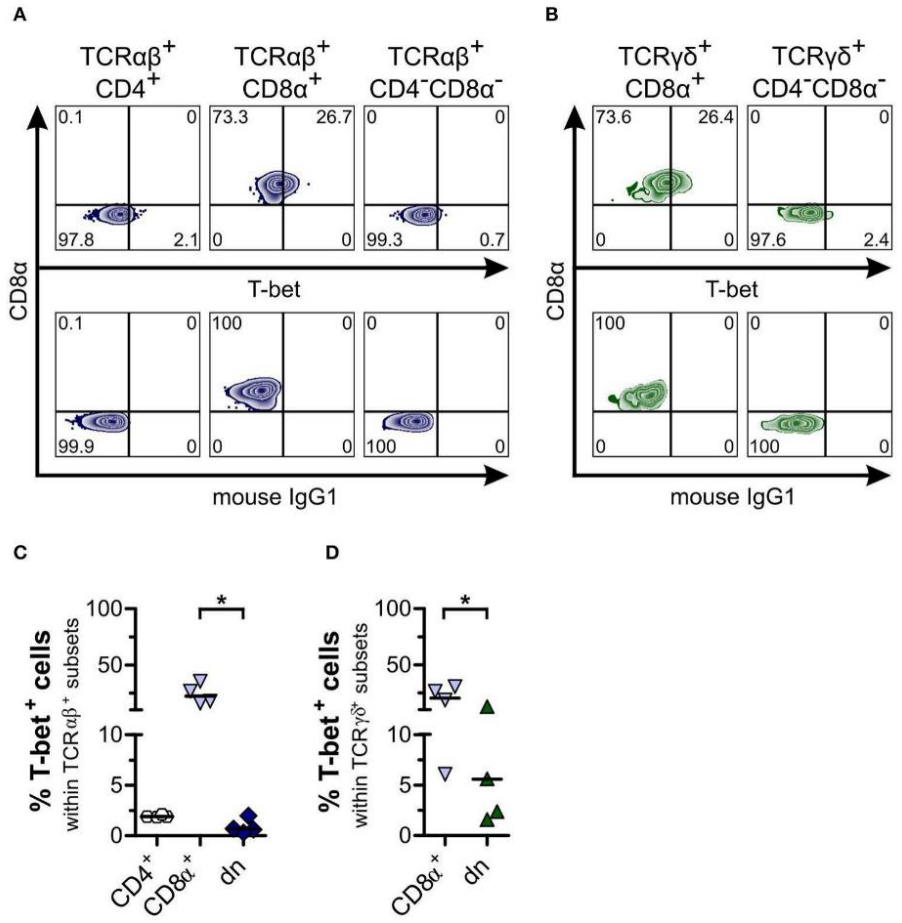

FIGURE 6 | Canine CD4⁻CD8α⁻ double-negative (dn) T cells express low frequencies of T-bet in comparison to CD8α⁺ single-positive (sp) T cells. **(A)** Representative zebra plots illustrating expression of T-bet by TCRαβ⁺ T cells gated on CD4⁺ sp, CD8α⁺ sp, and CD4⁻CD8α⁻ dn PBMC (upper panels). **(B)** TCRγδ⁺ cells including CD8α⁺ sp and CD4⁻CD8α⁻ dn subsets were analyzed for T-bet expression shown by representative zebra plots (upper panels). For **(A,B)**, appropriate gating was confirmed by internal and Fluorescence Minus One controls (lower panels). **(C)** Frequencies of T-bet expression by TCRαβ⁺CD4⁺ sp (white hexagons), TCRαβ⁺CD8α⁺ sp (light blue triangles), and TCRαβ⁺CD4⁻CD8α⁻ dn (dark blue diamonds) T cells of PBMC are depicted. The horizontal bars indicate median values ($n = 4$). Statistical analysis was performed by One-way analysis of variance (ANOVA) with Dunn's Multiple Comparison Test (*$p < 0.05$). **(D)** Quantification of T-bet expression within TCRγδ⁺ T cell subsets (CD8α⁺, light blue triangles; CD4⁻CD8α⁻ dn, green triangles) is shown. The horizontal bars indicate mean values ($n = 4$). Statistical analysis was performed by unpaired Student's *t*-test (two-tailed, *$p < 0.05$).

CD4⁻CD8α⁻ dn T cells. FoxP3 is the master regulator of conventional regulatory T cells (Treg) (56). Indeed, FoxP3 expression by TCRαβ⁺CD4⁻CD8α⁻ dn T cells reached the same levels as found for classical Treg (i.e., TCRαβ⁺CD4⁺ sp T cells). Evidence for this regulatory potential might be the very recently described increase of peripheral blood CD4⁻CD8α⁻ dn T cells after food allergen-specific sublingual immunotherapy of dogs with adverse food reactions. A potential regulatory function of these cells was discussed, albeit FoxP3 expression was not analyzed (19). GATA-3 expression by Tregs has been shown to play an essential role for Treg function during inflammation but not at steady state (e.g., for recruitment of Tregs to inflamed sites and for maintenance of high levels of FoxP3 expression) (57). Thus, we analyzed potential co-expression of FoxP3 and GATA-3 by TCRαβ⁺CD4⁻CD8α⁻ dn T cells and were able to detect cells with simultaneous expression of FoxP3 and GATA-3. Co-expression of FoxP3 and GATA-3 by a subset of canine TCRαβ⁺CD4⁻CD8α⁻ dn T cells may stabilize their suppressive capacity and prevent conversion into a pro-inflammatory T helper (Th) 17 cell phenotype as has been shown for murine GATA-3-deficient Tregs (58). On the other hand, canine TCRαβ⁺ and TCRγδ⁺CD4⁻CD8α⁻ dn T cells showed a significantly elevated percentage of GATA-3⁺FoxP3⁻ cells as compared with TCRαβ⁺ resp. TCRγδ⁺CD8α⁺ sp T cells. TCRαβ⁺CD4⁻CD8α⁻ dn T cells reached even higher percentages of GATA-3 expression than TCRαβ⁺CD4⁺ sp Th cells. While low GATA-3 expression

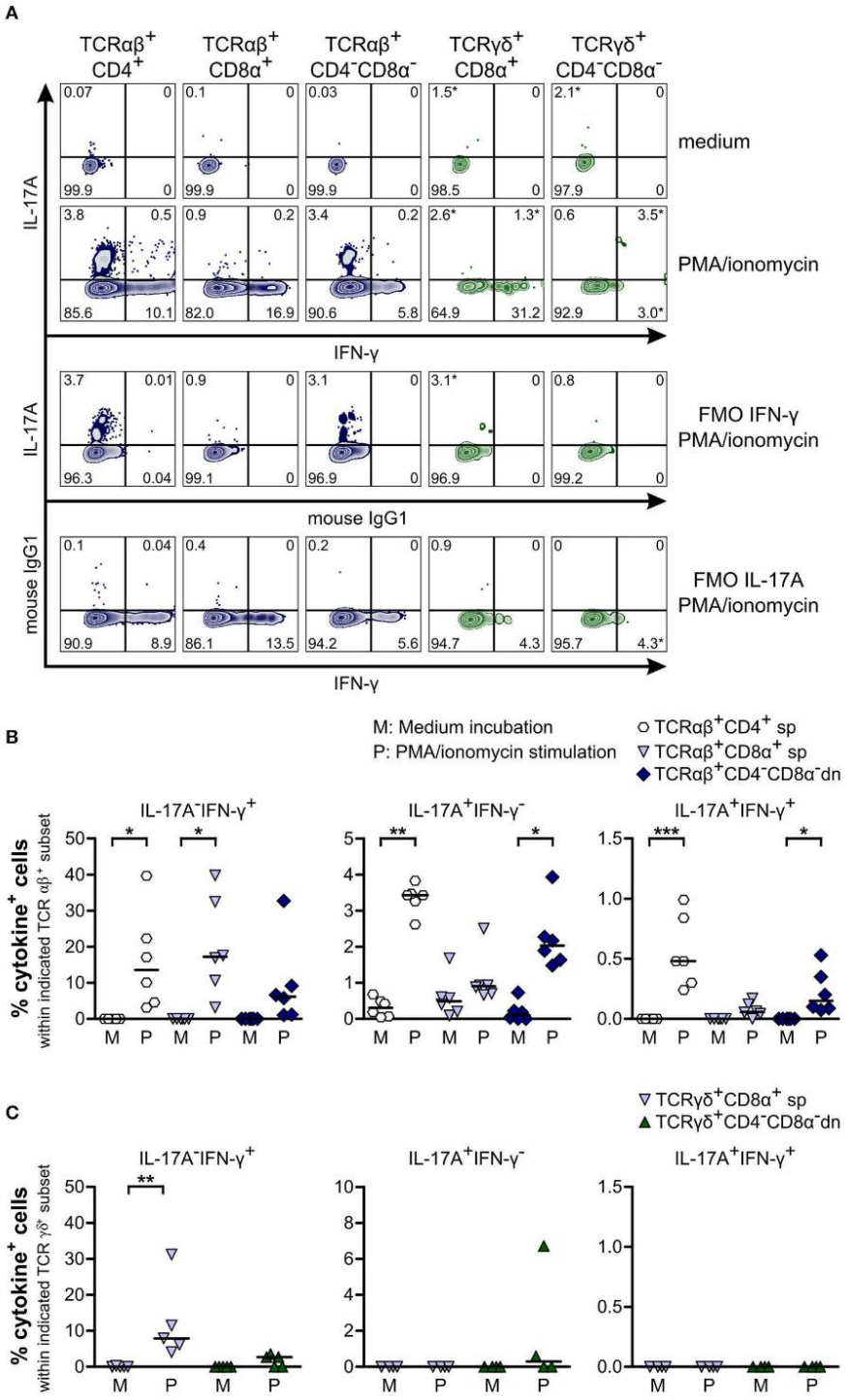

FIGURE 7 | Canine TCRαβ⁺CD4⁻CD8α⁻ but not TCRγδ⁺CD4⁻CD8α⁻ double-negative (dn) T cells produce IFN-γ and IL-17A upon PMA/ionomycin stimulation. **(A)** Representative zebra plots illustrating IL-17A and IFN-γ production by canine TCRαβ⁺ and TCRγδ⁺ T cell subsets after 4 h of PMA/ionomycin stimulation. Adequate

(Continued)

FIGURE 7 | gating was performed according to medium (upper panels) and Fluorescence Minus One (FMO) controls. Frequencies marked with * were disregarded based on the low MFI of only single data points according to Roederer (38). **(B)** Quantification of IL-17A and IFN-γ production by αβ T cell subsets after 4 h of medium (M) incubation or stimulation with PMA/ionomycin (P): $CD4^+$ sp (white hexagons), $CD8\alpha^+$ sp (light blue triangles), and $CD4^-CD8\alpha^-$ dn (dark blue diamonds) T cells. The horizontal bars indicate median values. Statistical analysis was performed by One-way analysis of variance (ANOVA) with Dunn's Multiple Comparison Test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Three independent experiments with $n = 6$ dogs in total were performed. **(C)** Quantification of IL-17A and IFN-γ production by $TCR\gamma\delta^+CD8\alpha^+$ sp (light blue triangles) and $TCR\gamma\delta^+CD4^-CD8\alpha^-$ dn (green triangles) T cells as described in **(B)**. The horizontal bars indicate median values. Statistical analysis was performed by One-way analysis of variance (ANOVA) with Dunn's Multiple Comparison Test (**$p < 0.01$). Three independent experiments with $n = 4$–5 dogs in total were performed.

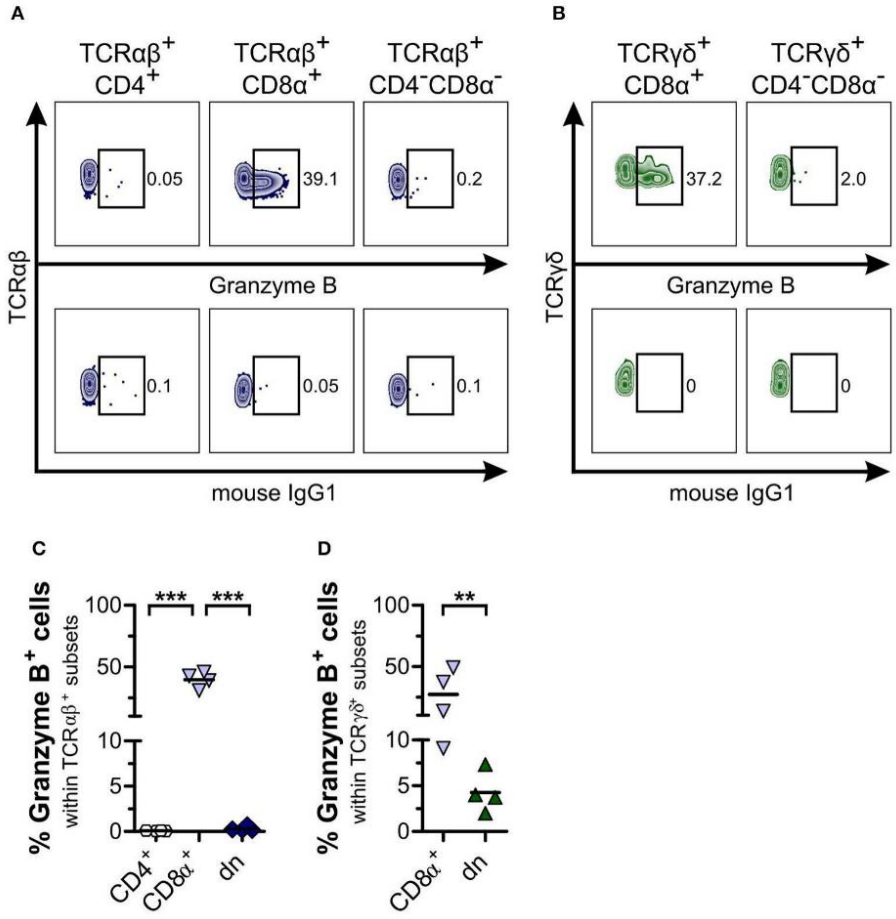

FIGURE 8 | $CD4^-CD8\alpha^-$ double-negative (dn) T cells are mainly granzyme B^-. **(A,B)** Representative zebra plots illustrating expression of granzyme B by $TCR\alpha\beta^+$ **(A)** resp. $TCR\gamma\delta^+$ **(B)** T cell subsets of PBMC (upper panels). Appropriate gating was confirmed by internal and Fluorescence Minus One controls (lower panels). Numbers in plots imply percentages. **(C,D)** The mean frequencies of granzyme B expression by $TCR\alpha\beta^+$ (CD4: white hexagons, $CD8\alpha^+$: light blue triangles, dn: dark blue diamonds) **(C)** resp. $TCR\gamma\delta^+$ ($CD8\alpha^+$: light blue triangles, dn: green triangles) **(D)** T cell subpopulations of PBMC are depicted. Each symbol represents one single dog ($n = 4$). For **(C)**, statistical analysis was performed by One-way analysis of variance (ANOVA) with Bonferroni's Multiple Comparison Test (***$p < 0.001$). For **(D)**, the unpaired Student's *t*-test (two-tailed, **$p < 0.01$) was applied.

(e.g., by $CD8\alpha^+$ sp T cells) may functionally be attributed to a role of GATA-3-dependent development of T cells (59), the high GATA-3 levels by $TCR\alpha\beta^+CD4^+$ sp T cells and in particular by $CD4^-CD8\alpha^-$ dn T cells may reflect the function of GATA-3 as Th2 master regulator (60, 61). Therefore, it is conceivable that canine $GATA\text{-}3^+CD4^-CD8\alpha^-$ dn T cells play a role in type 2 immunity such as anti-parasite responses or in the pathophysiology of allergy. To fully assess a potential type 2 regulatory function of canine $GATA\text{-}3^+CD4^-CD8\alpha^-$ dn T cells, characteristic features of Th2 cells such as production of IL-4, IL-5, or IL-13 need to be studied in future experiments. Besides, to verify a potential suppressive function of canine $FoxP3^+$

(GATA-3$^+$) TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells, their potential production of IL-10 and TGF-β as well as their suppressive capacity has to be assessed *in vitro*.

Unfortunately, due to the lack of cross-reactive monoclonal antibodies against canine RORγt, analysis of a potential Th17 transcriptional signature of canine CD4$^-$CD8α$^-$ dn T cells is currently not possible. However, we found evidence for a potential Th17 phenotype of a TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cell subset by analyzing expression of the cytokine IL-17A. In healthy humans, too, IL-17 has been found to be expressed by TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells. Increased frequencies of these cells were found in samples of human patients with autoimmune diseases (62–64). Furthermore, canine γδ T cells do not appear to be potent IL-17A producers. This is in clear contrast to murine, human, porcine and bovine γδ T cells (47–49, 51). It should be taken in account that canine γδ T cells might require different stimuli than PMA/ionomycin to produce IL-17, even though PMA/ionomycin was successfully used to stimulate γδ T cells of other species, e.g., swine (49, 65).

Further functional analysis of both subsets (TCRαβ$^+$ and TCRγδ$^+$) of canine CD4$^-$CD8α$^-$ dn T cells will help to generate new hypotheses related to their role *in vivo*. Besides, it should be validated whether TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells acquire cytotoxic potential upon activation despite their lack of granzyme B expression in the resting state.

With the features of CD4$^-$CD8α$^-$ dn T cell subsets described in our study, it is conceivable that they may have a pivotal function during homeostasis by suppressing exuberant immune responses and during inflammatory diseases of dogs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Usage Committee of the Saxony State Office (Landesdirektion Sachsen) in Leipzig, Germany (approval numbers: A 10/14 and A 28/18) (blood samples). Tissue samples: the study was approved by the Regierungspräsidium Darmstadt, Germany, approval number V54-19c 20/15-DA4/Anz.1004.

AUTHOR CONTRIBUTIONS

FR and ME: designed experiments, performed experiments, and wrote the manuscript. PM: provided reagents. KR, HB, DB, MB, and GA: wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02748/full#supplementary-material

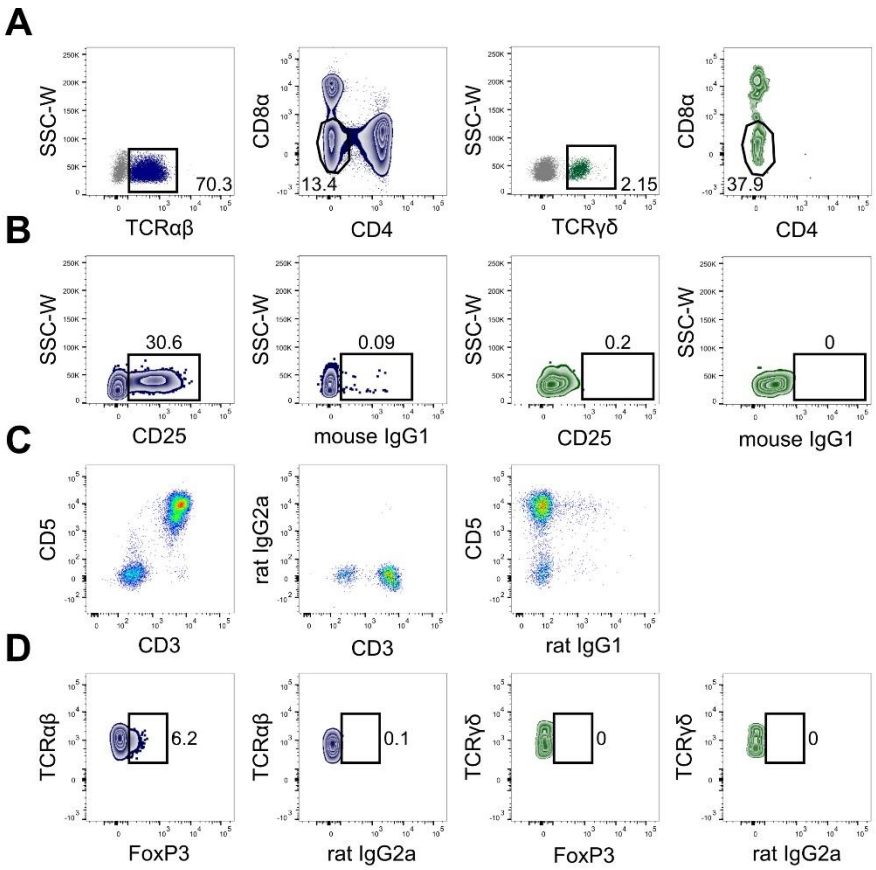
REFERENCES

1. Kol A, Arzi B, Athanasiou KA, Farmer DL, Nolta JA, Rebhun RB, et al. Companion animals: translational scientist's new best friends. *Sci Transl Med.* (2015) 7:308ps21. doi: 10.1126/scitranslmed.aaa9116
2. Mueller RS, Jensen-Jarolim E, Roth-Walter F, Marti E, Janda J, Seida AA, et al. Allergen immunotherapy in people, dogs, cats and horses - differences, similarities and research needs. *Allergy.* (2018) 73:1989–99. doi: 10.1111/all.13464
3. Hillhouse EE, Lesage S. A comprehensive review of the phenotype and function of antigen-specific immunoregulatory double negative T cells. *J Autoimmun.* (2013) 40:58–65. doi: 10.1016/j.jaut.2012.07.010
4. Scott CS, Richards SJ, Roberts BE. Patterns of membrane TcR alpha beta and TcR gamma delta chain expression by normal blood CD4+CD8-, CD4-CD8+, CD4-CD8dim+ and CD4-CD8- lymphocytes. *Immunology.* (1990) 70:351–6.
5. Singer PA, Theofilopoulos AN. Novel origin of lpr and gld cells and possible implications in autoimmunity. *J Autoimmun.* (1990) 3:123–35. doi: 10.1016/0896-8411(90)90136-G
6. Saalmüller A, Reddehase MJ, Bühring HJ, Jonjić S, Koszinowski UH. Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. *Eur J Immunol.* (1987) 17:1297–301. doi: 10.1002/eji.1830170912
7. Saalmüller A, Hirt W, Reddehase MJ. Phenotypic discrimination between thymic and extrathymic CD4-CD8- and CD4+CD8+ porcine T lymphocytes. *Eur J Immunol.* (1989) 19:2011–6. doi: 10.1002/eji.1830191107
8. Saalmüller A, Hirt W, Reddehase MJ. Porcine gamma/delta T lymphocyte subsets differing in their propensity to home to lymphoid tissue. *Eur J Immunol.* (1990) 20:2343–6. doi: 10.1002/eji.1830201026
9. Hirt W, Saalmüller A, Reddehase MJ. Distinct gamma/delta T cell receptors define two subsets of circulating porcine CD2-CD4-CD8- T lymphocytes. *Eur J Immunol.* (1990) 20:265–9. doi: 10.1002/eji.1830200206
10. Mackay CR, Maddox JF, Brandon MR. Three distinct subpopulations of sheep T lymphocytes. *Eur J Immunol.* (1986) 16:19–25. doi: 10.1002/eji.1830160105

11. Mackay CR, Beya MF, Matzinger P. Gamma/delta T cells express a unique surface molecule appearing late during thymic development. *Eur J Immunol.* (1989) 19:1477–83. doi: 10.1002/eji.1830190820
12. Giegerich GW, Hein WR, Miyasaka M, Tiefenthaler G, Hünig T. Restricted expression of CD2 among subsets of sheep thymocytes and T lymphocytes. *Immunology.* (1989) 66:354–61.
13. Clevers H, Machugh ND, Bensaid A, Dunlap S, Baldwin CL, Kaushal A, et al. Identification of a bovine surface antigen uniquely expressed on CD4–CD8– T cell receptor γ/δ+ T lymphocytes. *Eur J Immunol.* (1990) 20:809–17. doi: 10.1002/eji.1830200415
14. Sowder JT, Chen C-LH, Ager LL, Chan MM, Cooper MD. A large subpopulation of avian T cells express a homologue of the mammalian T gamma/delta receptor. *J Exp Med.* (1988) 167:315–22. doi: 10.1084/jem.167.2.315
15. Holderness J, Hedges JF, Ramstead A, Jutila MA. Comparative biology of γδ T cell function in humans, mice, and domestic animals. *Annu Rev Anim Biosci.* (2013) 1:99–124. doi: 10.1146/annurev-animal-031412-103639
16. Faldyna M, Sinkora J, Knotigova P, Leva L, Toman M. Lymphatic organ development in dogs: major lymphocyte subsets and activity. *Vet Immunol Immunopathol.* (2005) 104:239–47. doi: 10.1016/j.vetimm.2004.12.002
17. Pinheiro D, Singh Y, Grant CR, Appleton RC, Sacchini F, Walker KR, et al. Phenotypic and functional characterization of a CD4(+) CD25(high) FOXP3(high) regulatory T-cell population in the dog. *Immunology.* (2011) 132:111–22. doi: 10.1111/j.1365-2567.2010.03346.x
18. Alexandre-Pires G, Brito MT de, Algueró C, Martins C, Rodrigues OR, da Fonseca IP, et al. Canine leishmaniasis. Immunophenotypic profile of leukocytes in different compartments of symptomatic, asymptomatic and treated dogs. *Vet Immunol Immunopathol.* (2010) 137:275–83. doi: 10.1016/j.vetimm.2010.06.007
19. Maina E, Devriendt B, Cox E. Food allergen-specific sublingual immunotherapy modulates peripheral T cell responses of dogs with adverse food reactions. *Vet Immunol Immunopathol.* (2019) 212:38–42. doi: 10.1016/j.vetimm.2019.05.003
20. D'Acquisto F, Crompton T. CD3+CD4-CD8- (double negative) T cells: saviours or villains of the immune response? *Biochem Pharmacol.* (2011) 82:333–40. doi: 10.1016/j.bcp.2011.05.019
21. Thomson CW, Lee BP-L, Zhang L. Double-negative regulatory T cells: non-conventional regulators. *Immunol Res.* (2006) 35:163–78. doi: 10.1385/IR:35:1:163
22. Telfer JC, Baldwin CL. Bovine gamma delta T cells and the function of gamma delta T cell specific WC1 co-receptors. *Cell Immunol.* (2015) 296:76–86. doi: 10.1016/j.cellimm.2015.05.003
23. Hsu H, Chen C, Nenninger A, Holz L, Baldwin CL, Telfer JC. WC1 is a hybrid γδ TCR coreceptor and pattern recognition receptor for pathogenic bacteria. *J Immunol.* (2015) 194:2280–8. doi: 10.4049/jimmunol.1402021
24. Rodríguez-Gómez IM, Talker SC, Käser T, Stadler M, Reiter L, Ladinig A, et al. Expression of T-bet, eomesodermin, and GATA-3 correlates with distinct phenotypes and functional properties in porcine γδ T cells. *Front Immunol.* (2019) 10:396. doi: 10.3389/fimmu.2019.00396
25. Luckschander N, Pfammatter NS, Sidler D, Jakob S, Burgener IA, Moore PF, et al. Phenotyping, functional characterization, and developmental changes in canine intestinal intraepithelial lymphocytes. *Vet Res.* (2009) 40:58. doi: 10.1051/vetres/2009042
26. Rabiger FV, Bismarck D, Protschka M, Köhler G, Moore PF, Büttner M, et al. Canine tissue-associated CD4+CD8α+ double-positive T cells are an activated T cell subpopulation with heterogeneous functional potential. *PLoS ONE.* (2019) 14:e0213597. doi: 10.1371/journal.pone.0213597
27. Rothe K, Bismarck D, Büttner M, Alber G, Buttlar H von. Canine peripheral blood CD4+CD8+ double-positive Tcell subpopulations exhibit distinct Tcell phenotypes and effector functions. *Vet Immunol Immunopathol.* (2017) 185:48–56. doi: 10.1016/j.vetimm.2017.01.005
28. Mizuno T, Suzuki R, Umeki S, Okuda M. Crossreactivity of antibodies to canine CD25 and Foxp3 and identification of canine CD4+CD25+Foxp3+ cells in canine peripheral blood. *J Vet Med Sci.* (2009) 71:1561–8. doi: 10.1292/jvms.001561
29. Junginger J, Schwittlick U, Lemensieck F, Nolte I, Hewicker-Trautwein M. Immunohistochemical investigation of Foxp3 expression in the intestine in healthy and diseased dogs. *Vet Res.* (2012) 43:23. doi: 10.1186/1297-9716-43-23
30. Lee S-H, Shin D-J, Kim Y, Kim C-J, Lee J-J, Yoon MS, et al. Comparison of phenotypic and functional characteristics between canine non-B, non-T natural killer lymphocytes and CD3+CD5dimCD21- cytotoxic large granular lymphocytes. *Front Immunol.* (2018) 9:841. doi: 10.3389/fimmu.2018.00841
31. Withers SS, Moore PF, Chang H, Choi JW, McSorley SJ, Kent MS, et al. Multi-color flow cytometry for evaluating age-related changes in memory lymphocyte subsets in dogs. *Dev Comp Immunol.* (2018) 87:64–74. doi: 10.1016/j.dci.2018.05.022
32. Pedersen LG, Castelruiz Y, Jacobsen S, Aasted B. Identification of monoclonal antibodies that cross-react with cytokines from different animal species. *Vet Immunol Immunopathol.* (2002) 88:111–22. doi: 10.1016/S0165-2427(02)00139-3
33. Hartley AN, Tarleton RL. Chemokine receptor 7 (CCR7)-expression and IFNγ production define vaccine-specific canine T-cell subsets. *Vet Immunol Immunopathol.* (2015) 164:127–36. doi: 10.1016/j.vetimm.2015.02.001
34. Moreira ML, Dorneles EM, Soares RP, Magalhães CP, Costa-Pereira C, Lage AP, et al. Cross-reactivity of commercially available anti-human monoclonal antibodies with canine cytokines: establishment of a reliable panel to detect the functional profile of peripheral blood lymphocytes by intracytoplasmic staining. *Acta Vet Scand.* (2015) 57:51. doi: 10.1186/s13028-015-0142-y
35. Ritt MG, Lindborg BA, O'Brien TD, Bisignano J, Modiano JF. Stimulation with concanavalin-A induces IL-17 production by canine peripheral T cells. *Vet Sci.* (2015) 2:43–51. doi: 10.3390/vetsci2020043
36. Haas E, Rütgen BC, Gerner W, Richter B, Tichy A, Galler A, et al. Phenotypic characterization of canine intestinal intraepithelial lymphocytes in dogs with inflammatory bowel disease. *J Vet Intern Med.* (2014) 28:1708–15. doi: 10.1111/jvim.12456
37. Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Lett.* (1990) 276:172–4. doi: 10.1016/0014-5793(90)80535-Q
38. Roederer M. How many events is enough? Are you positive? *Cytometry A.* (2008) 73:384–5. doi: 10.1002/cyto.a.20549
39. Gerner W, Käser T, Saalmüller A. Porcine T lymphocytes and NK cells – an update: the porcine immune system. *Dev Comp Immunol.* (2009) 33:310–20. doi: 10.1016/j.dci.2008.06.003
40. Fischer K, Voelkl S, Heymann J, Przybylski GK, Mondal K, Laumer M, et al. Isolation and characterization of human antigen-specific TCR alpha beta+ CD4(-)CD8- double-negative regulatory T cells. *Blood.* (2005) 105:2828–35. doi: 10.1182/blood-2004-07-2583
41. Ascon DB, Ascon M, Satpute S, Lopez-Briones S, Racusen L, Colvin RB, et al. Normal mouse kidneys contain activated and CD3+CD4–CD8– double-negative T lymphocytes with a distinct TCR repertoire. *J Leukoc Biol.* (2008) 84:1400–9. doi: 10.1189/jlb.0907651
42. Voelkl S, Gary R, Mackensen A. Characterization of the immunoregulatory function of human TCR-αβ+ CD4- CD8- double-negative T cells. *Eur J Immunol.* (2011) 41:739–48. doi: 10.1002/eji.201040982
43. Cobbold S, Metcalfe S. Monoclonal antibodies that define canine homologues of human CD antigens: summary of the First International Canine Leukocyte Antigen Workshop (CLAW). *Tissue Antigens.* (1994) 43:137–54. doi: 10.1111/j.1399-0039.1994.tb02315.x
44. Herzig CT, Waters RW, Baldwin CL, Telfer JC. Evolution of the CD163 family and its relationship to the bovine gamma delta T cell co-receptor WC1. *BMC Evol Biol.* (2010) 10:181. doi: 10.1186/1471-2148-10-181
45. Rodamilans B, Muñoz IG, Bragado-Nilsson E, Sarrias MR, Padilla O, Blanco FJ, et al. Crystal structure of the third extracellular domain of CD5 reveals the fold of a group B scavenger cysteine-rich receptor domain. *J Biol Chem.* (2007) 282:12669–77. doi: 10.1074/jbc.M611699200
46. Zhang ZX, Yang L, Young KJ, DuTemple B, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat Med.* (2000) 6:782–9. doi: 10.1038/77513
47. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol.* (2010) 10:479–89. doi: 10.1038/nri2800
48. Akitsu A, Iwakura Y. Interleukin-17-producing γδ T (γδ17) cells in inflammatory diseases. *Immunology.* (2018) 155:418–26. doi: 10.1111/imm.12993

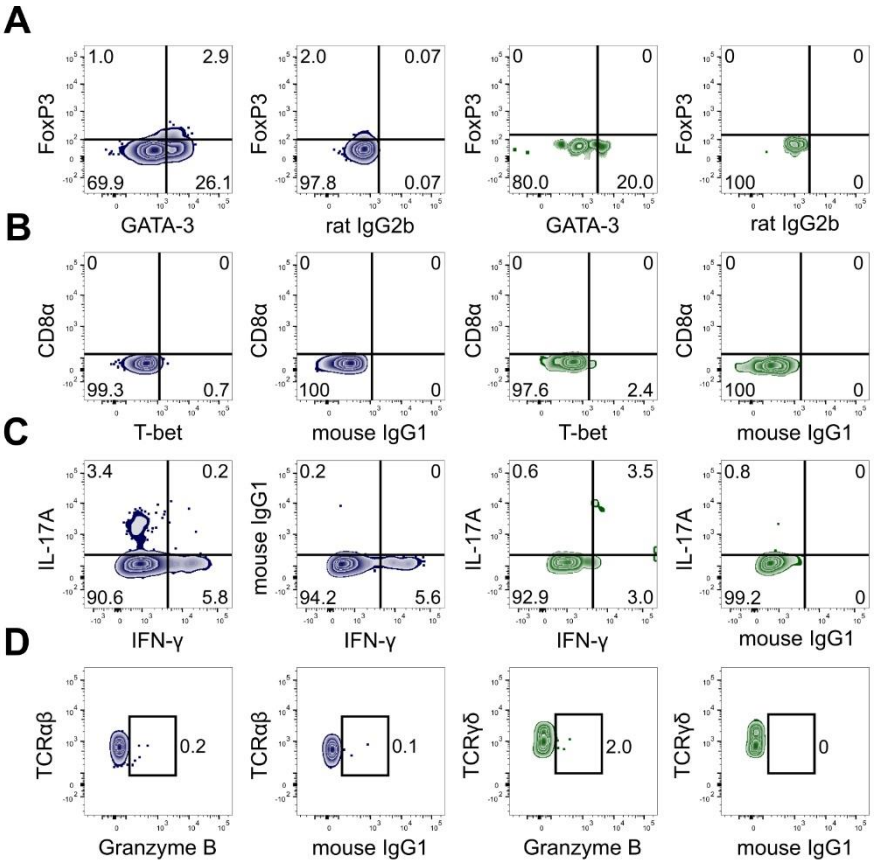
49. Sedlak C, Patzl M, Saalmüller A, Gerner W. CD2 and CD8α define porcine γδ T cells with distinct cytokine production profiles. *Dev Comp Immunol*. (2014) 45:97–106. doi: 10.1016/j.dci.2014.02.008
50. Mair KH, Sedlak C, Käser T, Pasternak A, Levast B, Gerner W, et al. The porcine innate immune system: an update. *Dev Comp Immunol*. (2014) 45:321–43. doi: 10.1016/j.dci.2014.03.022
51. Elnaggar MM, Abdellrazeq GS, Dassanayake RP, Fry LM, Hulubei V, Davis WC. Characterization of αβ and γδ T cell subsets expressing IL-17A in ruminants and swine. *Dev Comp Immunol*. (2018) 85:115–24. doi: 10.1016/j.dci.2018.04.003
52. Bismarck D, Schütze N, Moore P, Büttner M, Alber G, Buttlar Hv. Canine CD4+CD8+ double positive T cells in peripheral blood have features of activated T cells. *Vet Immunol Immunopathol*. (2012) 149:157–66. doi: 10.1016/j.vetimm.2012.06.014
53. Sato K, Ohtsuka K, Watanabe H, Asakura H, Abo T. Detailed characterization of gamma delta T cells within the organs in mice: classification into three groups. *Immunology*. (1993) 80:380–7.
54. Cowley SC, Hamilton E, Frelinger JA, Su J, Forman J, Elkins KL. CD4-CD8- T cells control intracellular bacterial infections both *in vitro* and *in vivo*. *J Exp Med*. (2005) 202:309–19. doi: 10.1084/jem.20050569
55. Johansson M, Lycke N. A unique population of extrathymically derived alpha beta TCR+CD4-CD8- T cells with regulatory functions dominates the mouse female genital tract. *J Immunol*. (2003) 170:1659–66. doi: 10.4049/jimmunol.170.4.1659
56. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. (2003) 4:330–6. doi: 10.1038/ni904
57. Wohlfert EA, Grainger JR, Bouladoux N, Konkel JE, Oldenhove G, Ribeiro CH, et al. GATA3 controls Foxp3 regulatory T cell fate during inflammation in mice. *J Clin Invest*. (2011) 121:4503–15. doi: 10.1172/JCI57456
58. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity*. (2011) 35:337–48. doi: 10.1016/j.immuni.2011.08.012
59. Tindemans I, Serafini N, Di Santo JP, Hendriks RW. GATA-3 function in innate and adaptive immunity. *Immunity*. (2014) 41:191–206. doi: 10.1016/j.immuni.2014.06.006
60. Zheng W-P, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell*. (1997) 89:587–96. doi: 10.1016/S0092-8674(00)80240-8
61. Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, et al. Conditional deletion of Gata3 shows its essential function in T H 1-T H 2 responses. *Nat Immunol*. (2004) 5:1157–65. doi: 10.1038/ni1128
62. Crispín JC, Tsokos GC. Human TCR-alpha beta+ CD4- CD8- T cells can derive from CD8+ T cells and display an inflammatory effector phenotype. *J Immunol*. (2009) 183:4675–81. doi: 10.4049/jimmunol.0901533
63. Crispín JC, Oukka M, Bayliss G, Cohen RA, van Beek CA, Stillman IE, et al. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol*. (2008) 181:8761–6. doi: 10.4049/jimmunol.181.12.8761
64. Alunno A, Bistoni O, Bartoloni E, Caterbi S, Bigerna B, Tabarrini A, et al. IL-17-producing CD4-CD8- T cells are expanded in the peripheral blood, infiltrate salivary glands and are resistant to corticosteroids in patients with primary Sjogren's syndrome. *Ann Rheum Dis*. (2013) 72:286–92. doi: 10.1136/annrheumdis-2012-201511
65. Stepanova H, Mensikova M, Chlebova K, Faldyna M. CD4+ and γδTCR+ T lymphocytes are sources of interleukin-17 in swine. *Cytokine*. (2012) 58:152–7. doi: 10.1016/j.cyto.2012.01.004

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



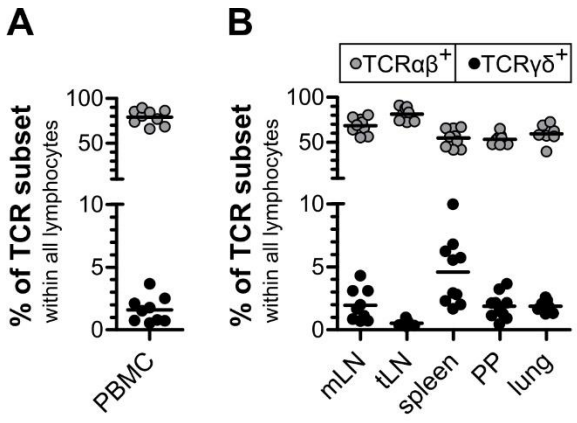
Supplemental Figure 1. Use of biexponential scaling in flow cytometry plots.

Representative flow cytometry plots showing use of biexponential scaling. (**A**) Non-doublet, viable lymphocytes were gated on TCRαβ$^+$ (first panel, blue) resp. TCRγδ$^+$ (third panel, green) CD4$^-$CD8α$^-$ double-negative (dn) T cells (second and fourth panels) as shown in Figure 1. **(B)** CD25 expression and corresponding Fluorescence Minus One (FMO) controls of TCRαβ$^+$CD4$^-$CD8α$^-$ (first and second panels) resp. TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells (third and fourth panels) as shown in Figure 2. **(C)** CD3/CD5 co-expression of canine living lymphocytes with corresponding FMO controls as shown in Figure 3. **(D)** FoxP3 expression and corresponding FMO controls of TCRαβ$^+$CD4$^-$CD8α$^-$ (first and second panels) resp. TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells (third and fourth panels) as shown in Figure 4.



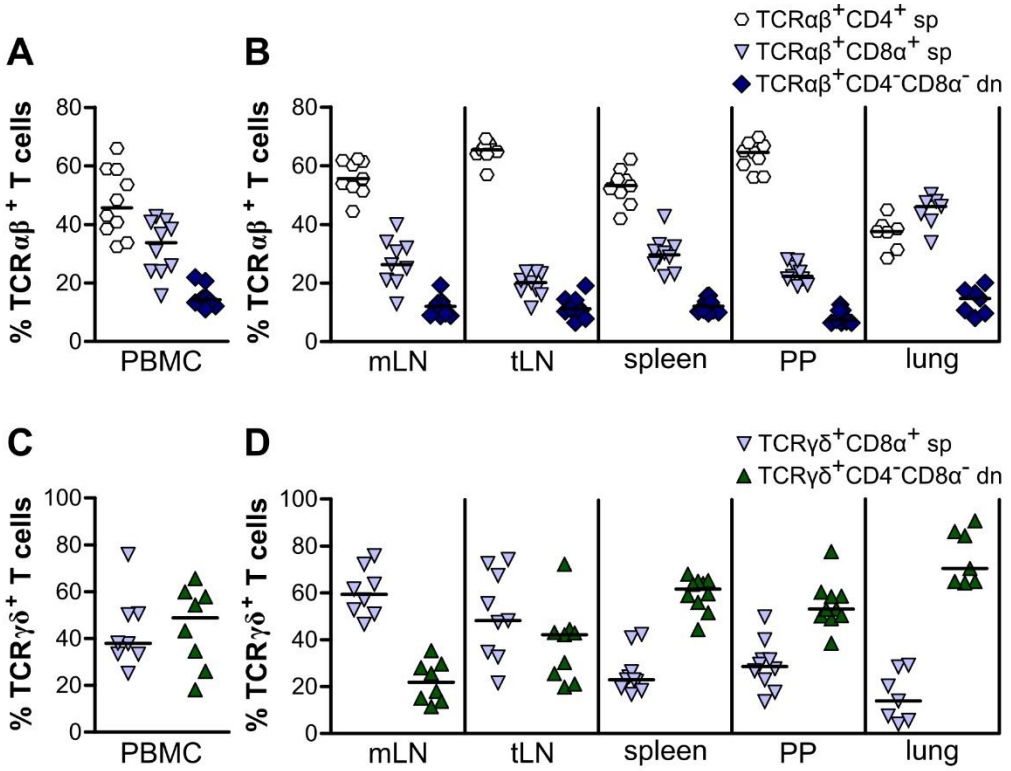
Supplemental Figure 2. Use of biexponential scaling in flow cytometry plots.

Representative flow cytometry plots showing use of biexponential scaling. **(A)** FoxP3/GATA-3 co-expression and corresponding Fluorescence Minus One (FMO) controls of TCRαβ$^+$CD4$^-$CD8α$^-$ (first and second panels) resp. TCRγδ$^+$CD4$^-$CD8α$^-$ double-negative (dn) T cells (third and fourth panels) as shown in Figure 5. **(B)** T-bet expression and corresponding FMO controls of TCRαβ$^+$CD4$^-$CD8α$^-$ (first and second panels) resp. TCRγδ$^+$CD4$^-$CD8α$^-$ dn cells (third and fourth panels) as shown in Figure 6. **(C)** IL-17A/IFN-γ expression and corresponding FMO controls of TCRαβ$^+$CD4$^-$CD8α$^-$ (first and second panels) resp. TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells (third and fourth panels) as shown in Figure 7. **(D)** Granzyme B expression and corresponding FMO controls of TCRαβ$^+$CD4$^-$CD8α$^-$ (first and second panels) resp. TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells (third and fourth panels) as shown in Figure 8.



Supplemental Figure 3. Quantification of TCRαβ$^+$ resp. TCRγδ$^+$ lymphocytes.

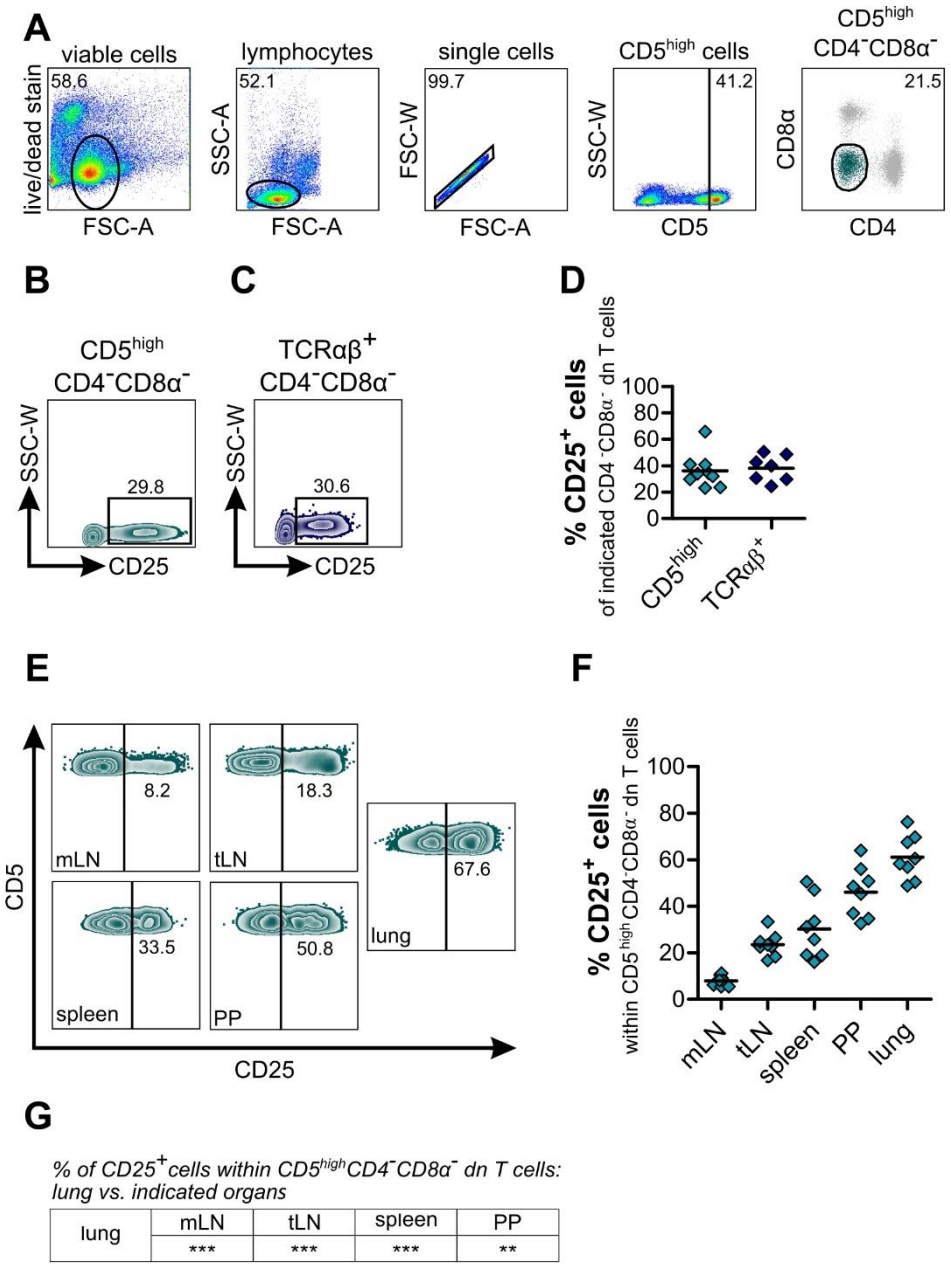
Non-doublet, viable lymphocytes of canine peripheral blood mononuclear cells (PBMC; **A**), mesenteric lymph nodes (mLN), tracheobronchial lymph nodes (tLN), spleen, Peyer's patches (PP), and lung **(B)** were gated on TCRαβ$^+$ (grey dots) resp. TCRγδ$^+$ (black dots) T cells. Each symbol represents one individual dog, the horizontal bars display mean values. Peripheral blood was taken from a different group of Beagle dogs (n = 9) than tissues (n = 7 – 10).



Supplemental Figure 4. Frequencies of CD4$^-$CD8α$^-$ double-negative (dn) in comparison to CD4$^+$ and CD8α$^+$ single-positive (sp) T cells.

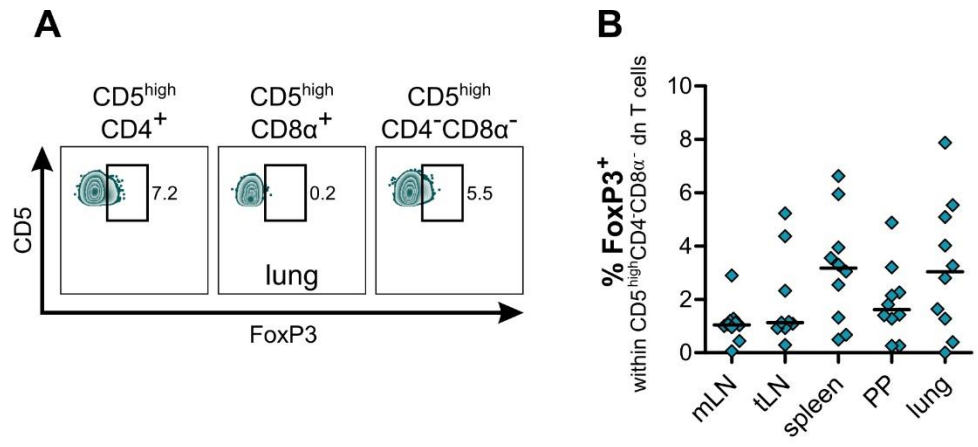
Quantification of TCRαβ$^+$CD4$^+$ sp (white hexagons), TCRαβ$^+$CD8α$^+$ sp (light blue triangles), and TCRαβ$^+$CD4$^-$CD8α$^-$ dn (dark blue diamonds) of canine PBMC **(A)**, mesenteric lymph nodes (mLN), tracheobronchial lymph nodes (tLN), spleen, Peyer's patches (PP), and lung **(B)** is shown. For TCRγδ$^+$ T cells, frequencies of TCRγδ$^+$CD8α$^+$ sp (light blue triangles) compared to TCRγδ$^+$CD4$^-$CD8α$^-$ dn (green triangles) T cells of PBMC **(C)** and indicated organs **(D)** are depicted.

Each symbol represents one individual dog, the horizontal bars display median values. Peripheral blood was taken from a different group of Beagle dogs (n = 8 – 10) than tissues (n = 7 – 10).



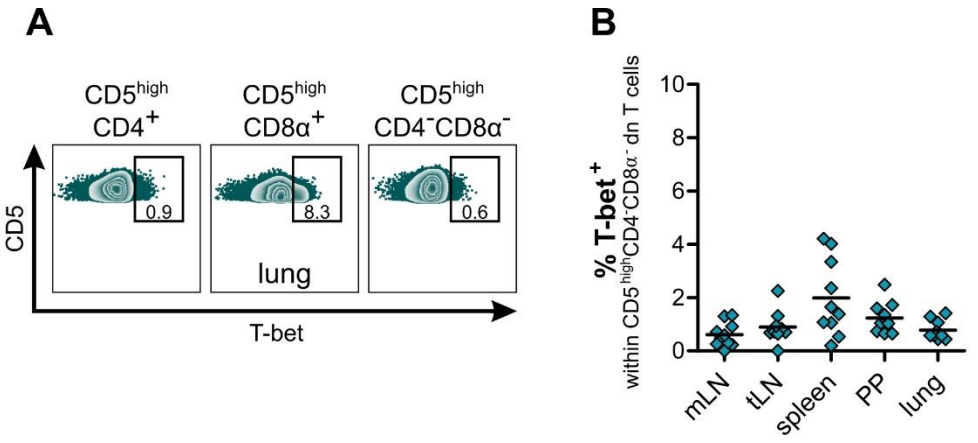
Supplemental Figure 5. CD25 expression of CD5^{high}CD4^{-}CD8α^{-} double-negative (dn) T cells.

(A) After exclusion of dead cells (left panel), gating on lymphocytes (second panel) was performed according to their forward and side scattering (FSC/SSC) properties. Following doublet-exclusion (FSC-W vs. FSC-A, middle panel), only CD5^{high}CD4^{-}CD8α^{-} dn T cells (fourth and fifth panel) were included into subsequent analyses. **(B + C)** Representative zebra plots of CD4^{-}CD8α^{-} dn T cells of PBMC pregated on CD5^{high} **(B)** or TCRαβ **(C)** are depicted. **(D)** Frequencies of CD25 expression of CD5^{high}CD4^{-}CD8α^{-} (light blue diamonds, n = 9) and TCRαβ^{+}CD4^{-}CD8α^{-} (dark blue diamonds, n = 7) dn T cells of PBMC are compared. Each symbol represents one single dog, frequencies are depicted with the mean. **(E)** Representative zebra plots illustrating CD25 expression of CD5^{high}CD4^{-}CD8α^{-} dn T cells from mesenteric lymph nodes (mLN), tracheobronchial lymph nodes (tLN), spleen, Peyer's patches (PP), and lung (n = 8 – 10). **(F + G)** The mean frequencies of CD25^{+} CD5^{high}CD4^{-}CD8α^{-} dn T cells of indicated organs are depicted. Each symbol represents one single dog. Statistical analysis was performed by One-way analysis of variance (ANOVA) with Bonferroni's Multiple Comparison Test (** $p < 0.01$, *** $p < 0.001$).



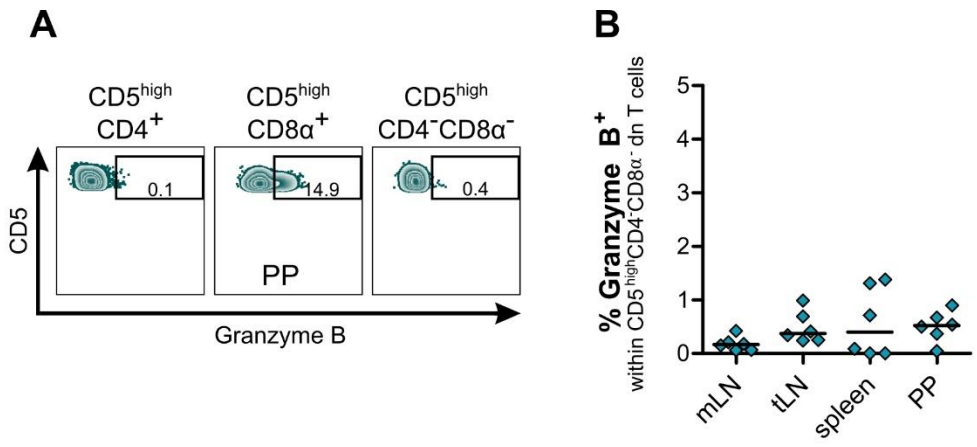
Supplemental Figure 6. FoxP3 expression of CD5^{high}CD4^{-}CD8α^{-} double-negative (dn) T cells within indicated organs.

(A) CD5^{high}CD4^{-}CD8α^{-} dn T cells were analyzed for expression of FoxP3 in comparison to CD5^{high}CD4^{+} single-positive (sp) and CD5^{high}CD8α^{+} sp T cells. Shown are zebra plots of lung standing representative for all analyzed organs in this study. The numbers in zebra plots imply percentages. **(B)** Quantification of FoxP3^{+} CD5^{high}CD4^{-}CD8α^{-} dn T cells from mesenteric lymph nodes (mLN), tracheobronchial lymph nodes (tLN), spleen, Peyer's patches (PP) and lung is shown. Each symbol represents one individual dog, the horizontal bars indicate median values (n = 9 – 10).



Supplemental Figure 7. T-bet expression of CD5^{high}CD4^{-}CD8α^{-} double-negative (dn) T cells within indicated organs.

(A) CD5^{high}CD4^{-}CD8α^{-} dn T cells were analyzed for T-bet expression in comparison to CD5^{high}CD4^{+} single-positive (sp) and CD5^{high}CD8α^{+} sp T cells. Shown are zebra plots of lung standing representative for all analyzed organs in this study. The numbers in zebra plots imply percentages. **(B)** Quantification of T-bet expression by CD5^{high}CD4^{-}CD8α^{-} dn T cells from mesenteric lymph nodes (mLN), tracheobronchial lymph nodes (tLN), spleen, Peyer's patches (PP) and lung is shown. Each symbol represents one individual dog, the horizontal bars indicate mean values (n = 9 – 10).



Supplemental Figure 8. Granzyme B expression of CD5^{high}CD4^{-}CD8α^{-} double-negative (dn) T cells within indicated organs.

(A) CD5^{high}CD4^{-}CD8α^{-} dn T cells were analyzed for expression of Granzyme B in comparison to CD5^{high}CD4^{+} single-positive (sp) and CD5^{high}CD8α^{+} sp T cells. Shown are zebra plots of Peyer's patches (PP) standing representative for mesenteric lymph nodes (mLN), tracheobronchial lymph nodes (tLN), and spleen. The numbers in zebra plots imply percentages. **(B)** Quantification of Granzyme B expression by CD5^{high}CD4^{-}CD8α^{-} dn T cells from indicated organs is shown. Each symbol represents one individual dog, the horizontal bars indicate mean values (n = 6).

3 Discussion

In the following, emphasis will be given to aspects which have not been discussed extensively in each of the two publications (see above): (i) tissue distribution of canine $CD4^+CD8\alpha^+$ dp T cells, (ii) potential role of canine $CD4^-CD8\alpha^-$ dn T cells.

3.1 Canine non-conventional $CD4^+CD8\alpha^+$ double-positive T cells are a homogeneous population enriched at mucosal sites

As described above, we found a homogeneous $TCR\alpha\beta^+CD4^+CD8\alpha\alpha^+CD25^+$ dp T cell population in canine spleen, duodenal/jejunal Peyer's patches (PP), and lung. While $CD4^+CD8\alpha^+$ dp T cells were very low in number and heterogeneous regarding expression levels of CD4 and CD8α in lymph nodes, they were most abundant in PP of the proximal small intestine. PP as part of the mucosa-associated lymphoid tissue (MALT) – or more precisely the gut-associated lymphoid tissue (GALT) – are considered as main site of IgA production. Being permanently exposed to a great variety of antigens of the gut lumen, PP take a unique position among all secondary lymphatic tissues (STEBEGG et al. 2018). This might be a reason for the accumulation of $CD4^+CD8\alpha^+$ dp T cells in canine PP. Interestingly, within canine tonsils, which are also lymphoid tissues highly exposed to antigens (BRANDTZAEG 2011), frequencies of $CD4^+CD8\alpha^+$ dp T cells are rather comparable to peripheral blood and lymph nodes than to PP (unpublished data). The question arises whether the gut microbial flora has an influence on the development of canine $CD4^+CD8\alpha^+$ dp T cells like it has been observed by SINKORA et al. 2011 for porcine $CD4^+CD8\alpha^+$ dp T cells from PP. This group compared the cellular composition of several porcine lymphoid organs in germ-free animals before and after colonization with apathogenic *Escherichia coli* resp. commensal gut flora. A significant increase in $CD4^+CD8\alpha^+$ dp T cells in bone marrow and PP was demonstrated after colonization of the intestine. Histologically, PP are organized into several regions: 1. The follicle-associated epithelium with M cells specialized on transcytosis of antigens; 2. The subepithelial dome dominated by dendritic cells taking up antigen from M cells and presenting them to B cells; 3. Large B cell follicles with germinal centers as main site of IgA production; 4. Small T cell zones next to the follicles (HOGENESCH et al. 1987, HOGENESCH and FELSBURG 1990, REBOLDI and CYSTER 2016). In addition to the T cell zones, where naïve T cells become activated following antigen encounter (ANDRIAN and MACKAY 2000), some T cells are scattered over the B cell follicles (HOGENESCH and FELSBURG 1992). As described above, these T cells were identified in human lymphoid tissues as C-X-C motif chemokine receptor 5 $(CXCR5)^+CD4^+$ follicular T helper (Tfh) specialized on B cell help with B cell lymphoma 6 protein (BCL6) as master transcription factor (reviewed by CROTTY 2019). Furthermore, $CD8\alpha^+CXCR5^+$ effector memory Tfh with anti-viral functions have been identified in humans, simians and mice (reviewed by PERDOMO-CELIS et al. 2017). Interestingly, murine Tfh of PP develop from $FoxP3^+$ Treg, thereby losing FoxP3 expression (TSUJI et al. 2009). This reveals similarities to the development of

murine lamina propria $CD4^+FoxP3^+$ Treg to $CD4^+CD8\alpha\alpha^+$ dp IEL which forfeit FoxP3 expression upon migration into the intestinal epithelium (SUJINO et al. 2016). Considering the striking differences in the function of T cells depending on their localization within lymphoid tissue, an immunohistological examination of canine Peyer's patches and other lymphoid organs would be useful. Immunofluorescent co-staining of CD4 and CD8α on canine tissue has recently been established by myself (data not shown) and can be used in subsequent experiments to provide evidence whether canine $CD4^+CD8\alpha^+$ dp T cells comprise subsets of Tfh. In addition, expression of BCL6 and CXCR5 in $CD4^+CD8\alpha^+$ dp T cells should be examined. While an anti-human-BCL6 antibody cross-reactive to dog tissue has already been successfully used by several groups (SATO et al. 2012, STEIN et al. 2019), expression of CXCR5 could be examined by RT-qPCR following sorting of $CD4^+CD8\alpha^+$ dp T cells.

Regarding $CD4^+CD8\alpha^+$ dp T cells of canine small intestinal IEL, our analyses concerning their frequency are consistent with the results of SONEA et al. 2000. However, only three-color flow cytometry was conducted in this study, whereby a more detailed analysis was not possible. We characterized canine small intestinal $CD4^+CD8\alpha^+$ dp IEL as $CD4^+CD8\alpha\alpha^+$ mainly expressing TCRαβ. This is in line with the phenotype of $CD4^+CD8\alpha^+$ dp IEL of mice (MORRISSEY et al. 1995, MOSLEY et al. 1990). Like $CD4^+CD8\alpha^+$ dp T cells of porcine Peyer's patches, these cells are absent in germ-free animals (MUCIDA et al. 2013, SUJINO et al. 2016) and can be induced by microbiota (CERVANTES-BARRAGAN et al. 2017). For humans, $TCR\alpha\beta^+CD4^+CD8\alpha\alpha^+$ dp IEL have also been described (JARRY et al. 1990, LATTHE et al. 1994). Though, MAYASSI and JABRI 2018 call for further characterization of human $TCR\alpha\beta^+CD4^+CD8\alpha^+$ dp IEL, as γδ T cells have not been excluded in the studies mentioned above (JARRY et al. 1990, LATTHE et al. 1994). The small intestinal epithelium of pigs also contains $TCR\alpha\beta^+CD4^+CD8\alpha^+$ dp IEL, yet the composition of the CD8 molecule has not yet been studied at this location (WIARDA et al. 2020). Canine IEL have already been thoroughly characterized by LUCKSCHANDER et al. 2009. The latter performed a comprehensive investigation of canine αβ and γδ IEL in neonates and adult dogs. However, $CD4^+CD8\alpha^+$ dp T cells were disregarded in this study (LUCKSCHANDER et al. 2009). As canine $CD4^+CD8\alpha^+$ dp T cells of peripheral blood increase with age (BISMARCK et al. 2012), one would have expected a higher portion of $CD4^+CD8\alpha^+$ dp T cells in their IEL samples of adult Beagle dogs (mean age 8.2 +/- 2.8 years) than ours (age range 0.8 - 1.2 years). Interestingly, the number of porcine $CD4^+CD8\alpha^+$ dp IEL decreases with age, whereas $CD8\alpha^+$ sp T cells increase (WIARDA et al. 2020). This is in contrast to porcine $CD4^+CD8\alpha^+$ dp T cells of peripheral blood (ZUCKERMANN 1999) and underlines the fact that intestinal intraepithelial lymphocytes do not follow the rules valid for T cells at other locations (OLIVARES-VILLAGÓMEZ and VAN KAER 2018). Accordingly, future studies focusing on canine $CD4^+CD8\alpha^+$ dp IEL partly expressing granzyme B or TCRγδ would be useful to determine their regulatory and/or effector functions.

Finally, we found a small, but distinct and homogeneous TCRαβ$^+$CD4$^+$CD8αα$^+$ dp T cell population in canine lungs. Lung leukocytes of dogs are scattered throughout the alveolar interstitium. An organized bronchus-associated lymphoid tissue (BALT), however, is absent under healthy conditions (PEETERS et al. 2005). This is in clear contrast to the canine GALT, which is always present in an organized structure independent of the health status (own observations, unpublished; GERMAN et al. 1999, HOGENESCH et al. 1987) and might be a reason for the much smaller CD4$^+$CD8α$^+$ dp T cell population in lungs than in PP. One wonders whether canine pulmonary diseases associated with BALT formation result in an increase of lung CD4$^+$CD8α$^+$ dp T cells, which should be the object of further research.

In summary, our study characterizes canine CD4$^+$CD8α$^+$ dp T cells as an activated T cell subpopulation enriched at mucosal sites and emphasizes the need for tissue investigation in addition to peripheral blood.

3.2 Canine non-conventional CD4$^-$CD8α$^-$ double-negative T cells are unique in abundance and phenotype

Table 1: Summarized characteristics of canine TCRαβ$^+$CD4$^-$CD8α$^-$ dn and γδ T cells of peripheral blood

	TCRαβ$^+$ CD4$^-$CD8α$^-$ dn	**TCRγδ$^+$ CD4$^-$CD8α$^-$ dn**	**TCRγδ$^+$ CD8α$^+$ sp**
CD25$^+$FoxP3$^-$	+	-	-
CD25$^+$FoxP3$^+$	+	-	-
GATA-3$^+$FoxP3$^+$	+	-	-
GATA-3$^+$FoxP3$^-$	+	+	-
IL-17A	+	-	-
IFN-γ	+	-	+
T-bet	low	low	+
Granzyme B	low	low	+

In our study on TCRαβ$^+$CD4$^-$CD8α$^-$ dn and γδ T cells, we found a much larger TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cell population in canine peripheral blood than it is known for mice and humans (HILLHOUSE and LESAGE 2013). Furthermore, to the best of our knowledge, this T cell subpopulation has not yet been comprehensively characterized in other domestic animals. Canine TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells consist of several subpopulations expressing key transcription factors or cytokines of their single-positive counterparts (summarized in table 1). First of all, the Treg-like CD25$^+$FoxP3$^+$ subpopulation should be mentioned. It is known already from mice and humans that TCRαβ$^+$CD4$^-$CD8α$^-$ dn

T cells of these species exhibit regulatory functions (D'ACQUISTO and CROMPTON 2011, HILL-HOUSE and LESAGE 2013). However, neither murine nor human TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells express FoxP3, the master transcription factor of CD4$^+$CD25$^+$ sp Treg (FISCHER et al. 2005, HILL-HOUSE et al. 2010, VOELKL et al. 2011, ZHANG et al. 2007). This reflects a remarkable difference to canine TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells, which clearly express this regulatory marker. Analyses of the cytokines IL-10 and TGF-β as well as *in vitro* suppression assays are necessary to confirm the hypothesis of canine suppressive TCRαβ$^+$CD4$^-$CD8α$^-$ Treg-like cells. Due to the lack of canine-specific or cross-reactive anti-IL-10 or anti-TGF-β-antibodies, single-cell RNA *in situ* hybridization in combination with flow cytometry would be a useful method to detect expression of these cytokines on a single-cell-level. First attempts to establish a protocol have been made by myself, whereas PINHEIRO et al. 2011 have already established an *in vitro* suppression assay for canine conventional CD4$^+$CD25$^+$FoxP3$^+$ Treg. This group already suspected but did not provide evidence for the existence of canine CD4$^-$CD8α$^-$ dn Treg and demanded further analyses. Among the FoxP3$^+$ Treg-like TCRαβ$^+$CD4$^-$CD8α$^-$ dn subpopulation we found a subset co-expressing GATA-3, the master transcription factor of Th2 cells (ZHENG and FLAVELL 1997, ZHU et al. 2004). As mentioned above, human or murine TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells are FoxP3$^-$ (FISCHER et al. 2005, HILLHOUSE et al. 2010, VOELKL et al. 2011, ZHANG et al. 2007).Thus, in humans FoxP3$^+$GATA-3$^+$ TCRαβ$^+$CD4$^-$CD8α$^-$ dn hybrid cells are lacking. However, CD4$^+$ sp FoxP3$^+$GATA-3$^+$ hybrid cells seem to play a crucial role in maintenance of the regulatory function of murine as well as human tissue Treg (WANG et al. 2011, WOHLFERT et al. 2011). In addition, the question arises whether this subset might selectively suppress Th2 immune responses, as RUDRA et al. 2012 demonstrated that murine Treg lacking GATA-3 were not able to do so. On the other hand, co-expression of GATA-3 in canine FoxP3$^+$ Treg-like TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells could imply an additional potential to exert type 2 immune responses when needed. A study investigating allergen-induced CD4$^+$FoxP3$^+$GATA-3$^+$ Treg in asthmatic children demonstrated production of IL-5 and IL-13 as well as suppressive capacity of this T cell subset (REUBSAET et al. 2013). Since allergies are of great importance in dog internal medicine (MARSELLA and BENEDETTO 2017), a further investigation of the FoxP3$^+$GATA3$^+$ hybrid as well as the large Th2-like FoxP3$^-$GATA-3$^+$ TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cell subset is needed. In addition to the aforementioned cytokines IL-10 and TGF-β, expression of IL-4, IL-5 and IL-13 by TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells should be the object of future research. Of note, human TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells are able to produce increased amounts of IL-4 in patients with SLE (DEAN et al. 2002). It is also known that TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells in human SLE patients contribute to the production of autoantibodies and secrete the pro-inflammatory cytokines IFN-γ and IL-17A (CRISPÍN et al. 2008, KATO and PERL 2014, LAI et al. 2013). Since we have shown that TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells of healthy dogs are also able to produce IFN-γ as well as IL-17A, it would be of high interest whether they are also involved in the pathogenesis of the canine variant of SLE. To this end, an analysis of the lymphocytic populations within the most affected canine organs,

i.e. joints, skin, and kidneys (FOURNEL et al. 1992), would be an informative supplement to our study. Accordingly, it is already known that TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells accumulate in human and murine kidneys (ASCON et al. 2008, MARTINA et al. 2016). Recently, murine kidney TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells have been studied for mechanisms of activation, revealing - similarly as for other T cell subsets - a crucial role of IL-2, but also their ability to respond to external stimuli in an MHC-independent manner. Furthermore, they seem to be dependent on non-classical β2-microglobulin (β2m) molecules for homeostasis (SADASIVAM et al. 2019). β2m molecules are also part of MR1, the receptor presenting microbial-derived vitamin B metabolites to MAIT cells (KELLER et al. 2017). MAIT cells are abundant in human peripheral blood and enriched at mucosal sites, such as the intestine (GODFREY et al. 2019). GHERARDIN et al. 2018 identified five subsets of human MAIT cells, thereof being 14.3% of CD4$^-$CD8α$^-$ dn and 1.3% of CD4$^+$CD8α$^+$ dp phenotype. Furthermore, human MAIT cells exhibit an effector phenotype and can produce IFN-γ, TNF-α, IL-2, IL-17A, and IL-22 (GHERARDIN et al. 2018). MAIT cells have also been identified in mice, cattle and pigs (GODFREY et al. 2019, GOLDFINCH et al. 2010, XIAO et al. 2019). Regarding the similarities to our findings in canine TCRαβ$^+$CD4$^-$CD8α$^-$ dn and – to a lesser extent – TCRαβ$^+$CD4$^+$CD8α$^+$ dp T cells, it is of particular interest whether canine non-conventional T cells comprise a subset of MAIT cells, too. Since MAIT cells have not yet been identified in the canine species, a first step would be the analysis of the T cell receptor repertoire of canine TCRαβ$^+$CD4$^-$CD8α$^-$ dn and TCRαβ$^+$CD4$^+$CD8α$^+$ dp T cells. For dogs, the TCRα/β repertoire could be analyzed by high-throughput sequencing using the 5'RACE (rapid amplification of cDNA ends) approach that was applied to characterize the canine immunoglobulin heavy chain repertoire before (HWANG et al. 2018). Since the majority of human TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cells has been shown to express a largely polyclonal TCR repertoire (FISCHER et al. 2005), I still expect MAIT cells to make up only a small fraction of the total canine TCRαβ$^+$CD4$^-$CD8α$^-$ dn T cell pool. Nevertheless, characterization of MAIT cells in dogs would be a milestone.

The small population of circulating canine γδ T cells comprises TCRγδ$^+$CD8α$^+$ sp as well as TCRγδ$^+$CD4$^-$CD8α$^-$ dn T cells. Canine TCRγδ$^+$CD8α$^+$ sp T cells resemble their TCRαβ$^+$ counterparts in regard to expression of T-bet, IFN-γ and granzyme B. This suggests comparable cytotoxic potential resp. Th1 polarization and should be verified by *in vitro* cytotoxicity assays in future studies. CD8α$^+$ γδ T cells are also present in chicken (BERNDT et al. 2006, GÖBEL et al. 1996) and swine. Interestingly, porcine CD8α$^+$ γδ T cells express perforin as well as T-bet (RODRÍGUEZ-GÓMEZ et al. 2019). The latter group also identified a large proportion of porcine GATA-3 expressing γδ T cells, being of a perforin$^-$CD8α$^{-/dim}$ phenotype that did not express IL-4. Consequently, they suspect this cellular population to be naïve. Similarly, we found a large subset of GATA-3$^+$CD4$^-$CD8α$^-$ dn T cells neither expressing CD25, T-bet or granzyme B within canine peripheral blood γδ T cells. Whether these cells are capable to produce Th2-associated cytokines (IL-4, IL-5, or IL-13) should be assessed as described above. In fact, OLIVRY et al. 1997 found increased numbers of epitheliotropic

γδ T cells in skin sections from dogs diagnosed with atopic dermatitis, suggesting an involvement of canine γδ T cells in the pathogenesis of allergies.

3.3 Concluding remarks

This is the first study providing an overview on canine non-conventional T cell subsets of peripheral blood resp. tissues. We identified several interesting subpopulations according to TCR and co-receptor distribution: Besides the conventional TCRαβ expressing $CD4^+$ and $CD8\alpha^+$ sp T cells, dogs possess $TCR\alpha\beta^+CD4^+CD8\alpha^+$ dp and $TCR\alpha\beta^+CD4^-CD8\alpha^-$ dn T cells as well as $CD8\alpha^+$ sp and $CD4^-CD8\alpha^-$ dn γδ T cells. Intraepithelial lymphocytes of the small intestine additionally contain $CD4^+CD8\alpha^+$ dp γδ T cells. Regarding this variety of T cell subpopulations comprising a broad range of functionally different subsets, it becomes clear that a detailed investigation of each subset is needed. A general conclusion about the role of $CD4^+CD8\alpha^+$ dp or $CD4^-CD8\alpha^-$ dn T cells in the canine immune system is therefore not possible, even though I suspect them to be essential. Hence, the classical scheme of $CD4^+$ and $CD8\alpha^+$ sp T cells as main effector/regulatory T cells in dogs should be called into question.

4 Summary

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Tissue distribution and characteristics of canine non-conventional T cells

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64 pages, 2 publications, 27 figures, 3 tables, 172 references

Keywords: dog, canine non-conventional T cells, $CD4^+CD8\alpha^+$ double-positive, $CD4^-CD8\alpha^-$ double-negative, TCRαβ, TCRγδ, tissue distribution

Introduction: Conventional αβ T cells comprise the classical $CD4^+$ single-positive (sp) T helper (Th) resp. T regulatory (Treg) and $CD8\alpha\beta^+$ sp cytotoxic T cell subsets. However, further T cell subpopulations either expressing T cell receptor αβ (TCRαβ) or γδ (TCRγδ) occur in mammals. In dogs, only the highly activated $CD4^+CD8\alpha^+$ double-positive (dp) T cell population of peripheral blood has been characterized in detail. Yet, little is known about its distribution in lymphoid and non-lymphoid tissues. Furthermore, dogs possess αβ T cells neither expressing CD4 nor CD8α ($CD4^-CD8\alpha^-$ double-negative (dn) T cells) and γδ T cells, whose phenotype was still undefined.

Aims of study: The objective of this study was to provide an overview on the occurrence and phenotype of non-conventional T cells in canine peripheral blood and tissues. To this end, $CD4^+CD8\alpha^+$ dp, $TCR\alpha\beta^+CD4^-CD8\alpha^-$ dn, and γδ T cells were characterized regarding expression of surface markers, key transcription factors, and cytokines.

Animals, material and methods: Canine T lymphocytes from peripheral blood (sample size: n = 10), Peyer's patches (PP; n = 10), epithelium of the small intestine (IEL; n = 6), mesenteric lymph nodes (mLN; n = 10), tracheobronchial lymph nodes (tLN; n = 9), lung (n = 10), spleen (n = 10), and thymus (n = 10) were analyzed by flow cytometry. Due to the study design tissue samples had to be taken from a different group of healthy Beagle dogs (n = 12) than peripheral blood. $CD4^+CD8\alpha^+$ dp T cells were studied for their tissue distribution, expression of T cell receptor type and composition of the CD8 dimer (i.e. CD8αα homodimer vs. CD8αβ heterodimer), as well as expression of the activation marker CD25. To define the potential function(s) of $CD4^+CD8\alpha^+$ dp T cells in tissues, expression of the transcription factor forkhead box P3 (FoxP3) and the cytotoxic molecule granzyme B were analyzed.

Furthermore, tissues and blood were studied for distribution of $TCR\alpha\beta^+CD4^-CD8\alpha^-$ dn and γδ T cells. A comprehensive characterization of blood $TCR\alpha\beta^+CD4^-CD8\alpha^-$ dn and γδ T cells was made by investigation of the following markers: CD25 and CD5, FoxP3, GATA-3, T-box transcription factor TBX21 (T-bet), and granzyme B. Functional analyses were performed by examination of cytokine

production (interferon γ (IFN-γ) and interleukin 17A, IL-17A) following stimulation of cells with phorbol-myristate-acetate (PMA)/ionomycin. Normally distributed data sets were analyzed using One-way analysis of variance (ANOVA) with Bonferroni post hoc test (multiple groups) or unpaired Student's t-test (two-tailed; two groups). For nonparametric data, either Kruskal-Wallis H test with Dunn's post-test (multiple groups) or Mann-Whitney U test (two-tailed; two groups) was applied.

Results: Canine $CD4^+CD8\alpha^+$ dp T cells of tissues can be mainly described as $TCR\alpha\beta^+CD4^+CD8\alpha\alpha^+CD25^+$ accumulating in PP (1.6% on average). Although not homogeneous in expression levels of CD4 and CD8α and low in number (0.2% on average), $CD4^+CD8\alpha^+$ dp T cells of LN are partly $FoxP3^+$. This indicates regulatory potential of this subset. Some $CD4^+CD8\alpha^+$ dp T cells of IEL express TCRγδ and/or granzyme B, underlining the uniqueness of intestinal intraepithelial lymphocytes.

$TCR\alpha\beta^+CD4^-CD8\alpha^-$ dn T cells make up a substantial portion of all αβ T cells in blood (median 14.4%) and tissues (median 15% (lung) – 7.5% (PP)) with a remarkably high expression level of CD25. CD25 is either co-expressed with FoxP3 (reminiscent of a regulatory phenotype), or without FoxP3 (reminiscent of an effector phenotype). In addition, subsets expressing IFN-γ, GATA-3, or IL-17A suggest properties of conventional type 1 T helper cells (Th1), Th2, and Th17, respectively. Interestingly, $FoxP3^+GATA\text{-}3^+$ hybrid cells were found, too. Canine γδ T cells, on the other hand, are either $CD8\alpha^+$ sp or $CD4^-CD8\alpha^-$ dn. While $TCR\gamma\delta^+CD8\alpha^+$ sp T cells resemble their $TCR\alpha\beta^+CD8\alpha\beta^+$ cytotoxic T cell counterparts (T-bet^+, IFN-γ^+, granzyme B^+), $TCR\gamma\delta^+CD4^-CD8\alpha^-$ dn T cells express GATA-3, and only low levels of T-bet and granzyme B.

Conclusions: This is the first study providing a comprehensive overview on canine non-conventional T cell subsets. Regarding tissue distribution, abundance and functions resp. functional potential of these T cells, it is conceivable that they play a major role in health and diseases of dogs. Further studies examining this topic will be needed. Of note, the classical scheme of $CD4^+$ sp Th/Treg and $CD8\alpha\beta^+$ cytotoxic T cells as main effector/regulatory T cells in dogs should be reconsidered.

5 Zusammenfassung

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Gewebeverteilung und Charakteristika nichtkonventioneller T-Zellen des Hundes

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Schlüsselwörter: Hund, canine nichtkonventionelle T-Zellen, $CD4^+CD8\alpha^+$ doppelt-positiv, $CD4^-CD8\alpha^-$ doppelt-negativ, TCRαβ, TCRγδ, Gewebeverteilung

Einleitung: Zu den konventionellen αβ T-Zellen zählen die klassischen $CD4^+$ einfach-positiven T-Helfer (Th) bzw. regulatorischen T-Zellen (Treg) sowie $CD8\alpha\beta^+$ einfach-positive zytotoxische T-Zellen. Säugetiere besitzen jedoch noch weitere T-Zell-Subpopulationen, die entweder den T-Zell-Rezeptor αβ (TCRαβ) oder γδ (TCRγδ) exprimieren. Bei Hunden wurde bisher nur die hoch aktivierte $CD4^+CD8\alpha^+$ doppelt-positive (dp) T-Zellpopulation des peripheren Blutes detailliert charakterisiert. Über ihre Verteilung in lymphatischem und nicht lymphatischem Gewebe ist jedoch wenig bekannt. Darüber hinaus besitzen Hunde αβ T-Zellen, die weder CD4 noch CD8α ($CD4^-CD8\alpha^-$ doppelt-negative (dn) T-Zellen) exprimieren, sowie γδ T-Zellen, deren Phänotyp bisher noch nicht untersucht wurde.

Ziele der Studie: Ziel dieser Studie war es, einen Überblick über das Vorkommen und den Phänotyp von nichtkonventionellen T-Zellen im peripheren Blut und Geweben des Hundes zu bieten. Zu diesem Zweck wurden $CD4^+CD8\alpha^+$ dp, $TCR\alpha\beta^+CD4^-CD8\alpha^-$ dn und γδ T-Zellen hinsichtlich der Expression von Oberflächenmarkern, wichtigen Transkriptionsfaktoren und Zytokinen charakterisiert.

Tiere, Material und Methoden: Canine T-Zellen Zellen aus dem Blut (Stichprobenumfang: n = 10) sowie aus den Peyerschen Platten (PP; n = 10), dem Dünndarmepithel (IEL; n = 6), den mesenterialen Lymphknoten (mLN; n = 10), tracheobronchialen Lymphknoten (tLN; n = 9), der Lunge (n = 10), Milz (n = 10) und dem Thymus (n = 10) wurden mittels Durchflusszytometrie analysiert. Das Studiendesign erforderte, dass die Gewebeproben von einer anderen Gruppe gesunder Beagle-Hunde (n = 12) entnommen wurden als das periphere Blut. $CD4^+CD8\alpha^+$ dp T-Zellen wurden auf ihre Gewebeverteilung, die Expression des T-Zell-Rezeptortyps und die Zusammensetzung des CD8-Dimers (d.h. CD8αα Homodimer vs. CD8αβ Heterodimer) sowie die Expression des Aktivierungsmarkers CD25 untersucht. Um Hinweise auf die potenzielle(n) Funktion(en) von $CD4^+CD8\alpha^+$ dp T-Zellen in Geweben zu erhalten, wurde die Expression des Transkriptionsfaktors Forkhead Box P3 (FoxP3) und des zytotoxischen Moleküls Granzym B analysiert.

Darüber hinaus wurden Gewebe und Blut auf die Verteilung von TCRαβ$^+$CD4$^-$CD8α$^-$ dn und γδ T-Zellen untersucht. Eine umfassende Charakterisierung von TCRαβ$^+$CD4$^-$CD8α$^-$ dn und γδ T-Zellen des peripheren Blutes erfolgte durch die Untersuchung der folgenden Marker: CD25 und CD5, FoxP3, GATA-3, T-Box-Transkriptionsfaktor TBX21 (T-bet) und Granzym B. Im Rahmen funktioneller Analysen wurde die Zytokinproduktion (Interferon-γ (IFN-γ) und Interleukin-17A, IL-17A) nach Stimulation der Zellen mit Phorbol-Myristat-Acetat (PMA)/Ionomycin durchgeführt. Normalverteilte Datensätze wurden mittels einfaktorieller Varianzanalyse (ANOVA) mit Bonferroni-Post-Hoc-Test (Vergleich mehrerer Gruppen) oder ungepaartem t-Test (zweiseitig; Vergleich zweier Gruppen) analysiert. Bei nichtparametrischen Daten wurde entweder der Kruskal-Wallis H-Test mit Dunn's Post-Test (Vergleich mehrerer Gruppen) oder der Mann-Whitney U-Test (zweiseitig; Vergleich zweier Gruppen) angewandt.

Ergebnisse: Canine CD4$^+$CD8α$^+$ dp T-Zellen der Gewebe sind hauptsächlich TCRαβ$^+$CD4$^+$CD8αα$^+$CD25$^+$ und akkumulieren in den PP (durchschnittlich 1.6 %). Obwohl CD4$^+$CD8α$^+$ dp T-Zellen der LN CD4 und CD8α nicht gleich stark exprimieren und zahlenmäßig gering (durchschnittlich 0,2 %) sind, sind sie teilweise FoxP3$^+$. Dies weist auf ein regulatorisches Potential dieser Untergruppe hin. Einige CD4$^+$CD8α$^+$ dp T-Zellen der IEL exprimieren TCRγδ und/oder Granzym B, was die Einzigartigkeit der intestinalen intraepithelialen Lymphozyten unterstreicht.

TCRαβ$^+$CD4$^-$CD8α$^-$ dn T-Zellen machen einen wesentlichen Anteil aller αβ T-Zellen in Blut (Median 14,4 %) und Gewebe (Median 15 % (Lunge) - 7,5% (PP)) aus und besitzen ein bemerkenswert hohes Expressionsniveau an CD25. CD25 wird entweder mit FoxP3 (hinweisend auf einen regulatorischen Phänotyp) oder ohne FoxP3 (hinweisend auf einen Effektorphänotyp) exprimiert. Zusätzlich weisen IFN-γ, GATA-3 oder IL-17A-exprimierende Subpopulationen Eigenschaften von konventionellen Typ 1 T-Helferzellen (Th1), Th2 bzw. Th17 auf. Interessanterweise wurden auch FoxP3$^+$GATA-3$^+$-Hybridzellen gefunden. Canine γδ T-Zellen hingegen sind entweder CD8α$^+$ einfach-positiv oder CD4$^-$CD8α$^-$ dn. Während TCRγδ$^+$CD8α$^+$ einfach-positive T-Zellen ihren zytotoxischen TCRαβ$^+$CD8αβ$^+$ T-Zell-Pendants ähneln (T-bet$^+$, IFN-γ$^+$, Granzym B$^+$), exprimieren TCRγδ$^+$CD4$^-$CD8α$^-$ dn T-Zellen GATA-3 und nur geringe Mengen an T-bet und Granzym B.

Schlussfolgerungen: Dies ist die erste Studie, die einen umfassenden Überblick über nichtkonventionelle T-Zell-Populationen des Hundes bietet. Hinsichtlich ihrer Gewebeverteilung, ihrer Anzahl und ihres funktionellen Potentials ist es wahrscheinlich, dass diese Zellen an wichtigen Immunreaktionen sowie an der Pathogenese von Erkrankungen des Hundes beteiligt sind. Hierzu werden weitere Studien erforderlich sein. Es ist anzumerken, dass das klassische Schema der CD4$^+$ und CD8αβ$^+$ einfach-positiven T-Zellen als canine Haupteffektor- bzw. regulatorische T-Zellen neu überdacht werden sollte.

6 Literature Cited

Abbas AK, Trotta E, Simeonov DR, Marson A, Bluestone JA. Revisiting IL-2: Biology and therapeutic prospects. Sci Immunol 2018; 3(25) DOI: 10.1126/sciimmunol.aat1482.

Akari H, Terao K, Murayama Y, Nam KH, Yoshikawa Y. Peripheral blood CD4+CD8+ lymphocytes in cynomolgus monkeys are of resting memory T lineage. Int Immunol 1997; 9(4):591–7 DOI: 10.1093/intimm/9.4.591.

Akitsu A, Iwakura Y. Interleukin-17-producing γδ T (γδ17) cells in inflammatory diseases. Immunology 2018; 155(4):418–26 DOI: 10.1111/imm.12993.

Alexandre-Pires G, Brito MTV de, Algueró C, Martins C, Rodrigues OR, da Fonseca IP et al. Canine leishmaniasis. Immunophenotypic profile of leukocytes in different compartments of symptomatic, asymptomatic and treated dogs. Vet Immunol Immunopathol 2010; 137(3-4):275–83 DOI: 10.1016/j.vetimm.2010.06.007.

Andersen MH, Schrama D, Thor SP, Becker JC. Cytotoxic T cells. J Invest Dermatol 2006; 126(1):32–41 DOI: 10.1038/sj.jid.5700001.

Andrian UH von, Mackay CR. T-cell function and migration. Two sides of the same coin. N Engl J Med 2000; 343(14):1020–34 DOI: 10.1056/NEJM200010053431407.

Ascon DB, Ascon M, Satpute S, Lopez-Briones S, Racusen L, Colvin RB et al. Normal mouse kidneys contain activated and CD3+CD4−CD8− double-negative T lymphocytes with a distinct TCR repertoire. J Leukoc Biol 2008; 84(6):1400–9 DOI: 10.1189/jlb.0907651.

Berndt A, Pieper J, Methner U. Circulating gamma delta T cells in response to Salmonella enterica serovar enteritidis exposure in chickens. Infect Immun 2006; 74(7):3967–78 DOI: 10.1128/IAI.01128-05.

Bismarck D, Moore PF, Alber G, Buttlar H von. Canine CD4+CD8+ double-positive T cells can develop from CD4+ and CD8+ T cells. Vet Immunol Immunopathol 2014; 162(3-4):72–82 DOI: 10.1016/j.vetimm.2014.09.008.

Bismarck D, Schütze N, Moore P, Büttner M, Alber G, Buttlar H von. Canine CD4+CD8+ double positive T cells in peripheral blood have features of activated T cells. Vet Immunol Immunopathol 2012; 149(3-4):157–66 DOI: 10.1016/j.vetimm.2012.06.014.

Blue ML, Daley JF, Levine H, Craig KA, Schlossman SF. Biosynthesis and surface expression of T8 by peripheral blood T4+ cells in vitro. J Immunol 1986; 137(4):1202–7.

Bonilla FA, Oettgen HC. Adaptive immunity. J Allergy Clin Immunol 2010; 125(2 Suppl 2):S33-40 DOI: 10.1016/j.jaci.2009.09.017.

Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. Nat Rev Immunol 2010; 10(7):467–78 DOI: 10.1038/nri2781.

Brandt D, Hedrich CM. TCRαβ+CD3+CD4-CD8- (double negative) T cells in autoimmunity. Autoimmun Rev 2018; 17(4):422–30 DOI: 10.1016/j.autrev.2018.02.001.

Brandt D, Sergon M, Abraham S, Mäbert K, Hedrich CM. TCR+CD3+CD4−CD8− effector T cells in psoriasis. Clin Immunol 2017; 181:51–9 DOI: 10.1016/j.clim.2017.06.002.

Brandtzaeg P. Immune functions of nasopharyngeal lymphoid tissue. Adv Otorhinolaryngol 2011; 72:20–4 DOI: 10.1159/000324588.

Carding SR, Egan PJ. Gammadelta T cells: functional plasticity and heterogeneity. Nat Rev Immunol 2002; 2(5):336–45 DOI: 10.1038/nri797.

Cervantes-Barragan L, Chai JN, Tianero MD, Di Luccia B, Ahern PP, Merriman J et al. Lactobacillus reuteri induces gut intraepithelial CD4+CD8αα+ T cells. Science 2017; 357(6353):806–10 DOI: 10.1126/science.aah5825.

Chen W, Ford MS, Young KJ, Zhang L. Infusion of in vitro-generated DN T regulatory cells induces permanent cardiac allograft survival in mice. Transplant Proc 2003; 35(7):2479–80 DOI: 10.1016/j.transproceed.2003.08.030.

Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 1994; 265(5176):1237–40 DOI: 10.1126/science.7520605.

Cheroutre H, Lambolez F. Doubting the TCR coreceptor function of CD8alphaalpha. Immunity 2008; 28(2):149–59 DOI: 10.1016/j.immuni.2008.01.005.

Chetty R, Gatter K. CD3: structure, function, and role of immunostaining in clinical practice. J Pathol 1994; 173(4):303–7 DOI: 10.1002/path.1711730404.

Chien Y-h, Meyer C, Bonneville M. γδ T cells: first line of defense and beyond. Annu Rev Immunol 2014; 32:121–55 DOI: 10.1146/annurev-immunol-032713-120216.

Clark RA. Resident memory T cells in human health and disease. Sci Transl Med 2015; 7(269) DOI: 10.1126/scitranslmed.3010641.

Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: a dynamic protein ensemble. Annu Rev Immunol 1988; 6:629–62 DOI: 10.1146/annurev.iy.06.040188.003213.

Corthay A. How do regulatory T cells work? Scand J Immunol 2009; 70(4):326–36 DOI: 10.1111/j.1365-3083.2009.02308.x.

Crispín JC, Oukka M, Bayliss G, Cohen RA, van Beek CA, Stillman IE et al. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. J Immunol 2008; 181(12):8761–6 DOI: 10.4049/jimmunol.181.12.8761.

Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol 2011; 29:621–63 DOI: 10.1146/annurev-immunol-031210-101400.

Crotty S. T Follicular Helper Cell Biology: A Decade of Discovery and Diseases. Immunity 2019; 50(5):1132–48 DOI: 10.1016/j.immuni.2019.04.011.

D'Acquisto F, Crompton T. CD3+CD4-CD8- (double negative) T cells: saviours or villains of the immune response? Biochem Pharmacol 2011; 82(4):333–40 DOI: 10.1016/j.bcp.2011.05.019.

Dean GS, Anand A, Blofeld A, Isenberg DA, Lydyard PM. Characterization of CD3+ CD4- CD8- (double negative) T cells in patients with systemic lupus erythematosus: production of IL-4. Lupus 2002; 11(8):501–7 DOI: 10.1191/0961203302lu234oa.

Dieli F, Troye-Blomberg M, Ivanyi J, Fournié JJ, Krensky AM, Bonneville M et al. Granulysin-dependent killing of intracellular and extracellular Mycobacterium tuberculosis by Vgamma9/Vdelta2 T lymphocytes. J Infect Dis 2001; 184(8):1082–5 DOI: 10.1086/323600.

Dugas V, Beauchamp C, Chabot-Roy G, Hillhouse EE, Lesage S. Implication of the CD47 pathway in autoimmune diabetes. J Autoimmun 2010; 35(1):23–32 DOI: 10.1016/j.jaut.2010.01.002.

Duncan B, Nazarov-Stoica C, Surls J, Kehl M, Bona C, Casares S et al. Double negative (CD3+ 4-8-) TCR alphabeta splenic cells from young NOD mice provide long-lasting protection against type 1 diabetes. PLoS ONE 2010; 5(7):e11427 DOI: 10.1371/journal.pone.0011427.

Eyerich K, Dimartino V, Cavani A. IL-17 and IL-22 in immunity: Driving protection and pathology. Eur J Immunol 2017; 47(4):607–14 DOI: 10.1002/eji.201646723.

Faldyna M, Sinkora J, Knotigova P, Leva L, Toman M. Lymphatic organ development in dogs: major lymphocyte subsets and activity. Vet Immunol Immunopathol 2005; 104(3-4):239–47 DOI: 10.1016/j.vetimm.2004.12.002.

Finkelman FD, Hogan SP, Hershey GKK, Rothenberg ME, Wills-Karp M. Importance of cytokines in murine allergic airway disease and human asthma. J Immunol 2010; 184(4):1663–74 DOI: 10.4049/jimmunol.0902185.

Fischer K, Voelkl S, Heymann J, Przybylski GK, Mondal K, Laumer M et al. Isolation and characterization of human antigen-specific TCR alpha beta+ CD4(-)CD8- double-negative regulatory T cells. Blood 2005; 105(7):2828–35 DOI: 10.1182/blood-2004-07-2583.

Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003; 4(4):330–6 DOI: 10.1038/ni904.

Ford MS, Chen W, Wong S, Li C, Vanama R, Elford AR et al. Peptide-activated double-negative T cells can prevent autoimmune type-1 diabetes development. Eur J Immunol 2007; 37(8):2234–41 DOI: 10.1002/eji.200636991.

Fournel C, Chabanne L, Caux C, Faure JR, Rigal D, Magnol JP et al. Canine systemic lupus erythematosus. I: A study of 75 cases. Lupus 1992; 1(3):133–9 DOI: 10.1177/096120339200100303.

Galler A, Rütgen BC, Haas E, Saalmüller A, Hirt RA, Gerner W et al. Immunophenotype of Peripheral Blood Lymphocytes in Dogs with Inflammatory Bowel Disease. J Vet Intern Med 2017; 31(6):1730–9 DOI: 10.1111/jvim.14812.

Gangadharan D, Cheroutre H. The CD8 isoform CD8alphaalpha is not a functional homologue of the TCR co-receptor CD8alphabeta. Curr Opin Immunol 2004; 16(3):264–70 DOI: 10.1016/j.coi.2004.03.015.

Germain RN. T-cell development and the CD4-CD8 lineage decision. Nat Rev Immunol 2002; 2(5):309–22 DOI: 10.1038/nri798.

German AJ, Hall EJ, Day MJ. Analysis of leucocyte subsets in the canine intestine. J Comp Pathol 1999; 120(2):129–45 DOI: 10.1053/jcpa.1998.0262.

German AJ, Hall EJ, Moore PF, Ringler DJ, Newman W, Day MJ. The distribution of lymphocytes expressing alphabeta and gammadelta T-cell receptors, and the expression of mucosal addressin cell adhesion molecule-1 in the canine intestine. J Comp Pathol 1999; 121(3):249–63 DOI: 10.1053/jcpa.1999.0328.

Gerner W, Talker SC, Koinig HC, Sedlak C, Mair KH, Saalmüller A. Phenotypic and functional differentiation of porcine αβ T cells: Current knowledge and available tools. Mol Immunol 2015; 66(1):3–13 DOI: 10.1016/J.MOLIMM.2014.10.025.

Gherardin NA, Souter MN, Koay H-F, Mangas KM, Seemann T, Stinear TP et al. Human blood MAIT cell subsets defined using MR1 tetramers. Immunol Cell Biol 2018; 96(5):507–25 DOI: 10.1111/imcb.12021.

Gibson D, Aubert I, Woods JP, Abrams-Ogg A, Kruth S, Wood RD et al. Flow Cytometric Immunophenotype of Canine Lymph Node Aspirates. J Vet Int Med 2004; 18(5):710–7 DOI: 10.1111/j.1939-1676.2004.tb02610.x.

Göbel TW, Chen CH, Cooper MD. Expression of an avian CD6 candidate is restricted to alpha beta T cells, splenic CD8+ gamma delta T cells and embryonic natural killer cells. Eur J Immunol 1996; 26(8):1743–7 DOI: 10.1002/eji.1830260812.

Godfrey DI, Koay H-F, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. Nat Immunol 2019; 20(9):1110–28 DOI: 10.1038/s41590-019-0444-8.

Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. Nat Immunol 2015; 16(11):1114–23 DOI: 10.1038/ni.3298.

Goldfinch N, Reinink P, Connelley T, Koets A, Morrison I, van Rhijn I. Conservation of mucosal associated invariant T (MAIT) cells and the MR1 restriction element in ruminants, and abundance of MAIT cells in spleen. Vet Res 2010; 41(5):62 DOI: 10.1051/vetres/2010034.

Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, Vries JE de et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997; 389(6652):737–42 DOI: 10.1038/39614.

Guerra-Maupome M, Slate JR, McGill JL. Gamma Delta T Cell Function in Ruminants. Vet Clin North Am Food Anim Pract 2019; 35(3):453–69 DOI: 10.1016/j.cvfa.2019.08.001.

He KM, Ma Y, Wang S, Min W-P, Zhong R, Jevnikar A et al. Donor double-negative Treg promote allogeneic mixed chimerism and tolerance. Eur J Immunol 2007; 37(12):3455–66 DOI: 10.1002/eji.200737408.

Hillhouse EE, Beauchamp C, Chabot-Roy G, Dugas V, Lesage S. Interleukin-10 limits the expansion of immunoregulatory CD4-CD8- T cells in autoimmune-prone non-obese diabetic mice. Immunol Cell Biol 2010; 88(8):771–80 DOI: 10.1038/icb.2010.84.

Hillhouse EE, Lesage S. A comprehensive review of the phenotype and function of antigen-specific immunoregulatory double negative T cells. J Autoimmun 2013; 40:58–65 DOI: 10.1016/j.jaut.2012.07.010.

Hogenesch H, Felsburg PJ. Ultrastructure and alkaline phosphatase activity of the dome epithelium of canine Peyer's patches. Vet Immunol Immunopathol 1990; 24(2):177–86 DOI: 10.1016/0165-2427(90)90020-S.

Hogenesch H, Felsburg PJ. Immunohistology of Peyer's patches in the dog. Vet Immunol Immunopathol 1992; 30(2-3):147–60 DOI: 10.1016/0165-2427(92)90135-D.

HogenEsch H, Housman JM, Felsburg PJ. Canine Peyer's patches: macroscopic, light microscopic, scanning electron microscopic and immunohistochemical investigations. Adv Exp Med Biol 1987; 216A:249–56 DOI: 10.1007/978-1-4684-5344-7_29.

Hori S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. Science 2003; 299(5609):1057–61 DOI: 10.1126/science.1079490.

Hwang M-H, Darzentas N, Bienzle D, Moore PF, Morrison J, Keller SM. Characterization of the canine immunoglobulin heavy chain repertoire by next generation sequencing. Vet Immunol Immunopathol 2018; 202:181–90 DOI: 10.1016/j.vetimm.2018.07.002.

Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006; 126(6):1121–33 DOI: 10.1016/j.cell.2006.07.035.

Jameson SC, Masopust D. Understanding Subset Diversity in T Cell Memory. Immunity 2018; 48(2):214–26 DOI: 10.1016/j.immuni.2018.02.010.

Janeway CA, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002; 20:197–216 DOI: 10.1146/annurev.immunol.20.083001.084359.

Jarry A, Cerf-Bensussan N, Brousse N, Selz F, Guy-Grand D. Subsets of CD3+ (T cell receptor alpha/beta or gamma/delta) and CD3- lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. Eur J Immunol 1990; 20(5):1097–103 DOI: 10.1002/eji.1830200523.

Jasenosky LD, Scriba TJ, Hanekom WA, Goldfeld AE. T cells and adaptive immunity to Mycobacterium tuberculosis in humans. Immunol Rev 2015; 264(1):74–87 DOI: 10.1111/imr.12274.

Johansson M, Lycke N. A unique population of extrathymically derived alpha beta TCR+CD4-CD8- T cells with regulatory functions dominates the mouse female genital tract. J Immunol 2003; 170(4):1659–66 DOI: 10.4049/jimmunol.170.4.1659.

Junttila IS. Tuning the Cytokine Responses: An Update on Interleukin (IL)-4 and IL-13 Receptor Complexes. Front Immunol 2018; 9:888 DOI: 10.3389/fimmu.2018.00888.

Kaplan MH, Hufford MM, Olson MR. The development and in vivo function of T helper 9 cells. Nat Rev Immunol 2015; 15(5):295–307 DOI: 10.1038/nri3824.

Kato H, Perl A. Mechanistic target of rapamycin complex 1 expands Th17 and IL-4+ CD4-CD8- double-negative T cells and contracts regulatory T cells in systemic lupus erythematosus. J Immunol 2014; 192(9):4134–44 DOI: 10.4049/jimmunol.1301859.

Keller AN, Corbett AJ, Wubben JM, McCluskey J, Rossjohn J. MAIT cells and MR1-antigen recognition. Curr Opin Immunol 2017; 46:66–74 DOI: 10.1016/j.coi.2017.04.002.

Kelly K, Pilarski L, Shortman K, Scollay R. CD4+ CD8+ cells are rare among in vitro activated mouse or human T lymphocytes. Cell Immunol 1988; 117(2):414–24 DOI: 10.1016/0008-8749(88)90130-X.

Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol 2003; 4(4):337–42 DOI: 10.1038/ni909.

Koch U, Radtke F. Mechanisms of T cell development and transformation. Annu Rev Cell Dev Biol 2011; 27:539–62 DOI: 10.1146/annurev-cellbio-092910-154008.

König R. Interactions between MHC molecules and co-receptors of the TCR. Curr Opin Immunol 2002; 14(1):75–83 DOI: 10.1016/S0952-7915(01)00300-4.

Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Köhler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature 1993; 362(6417):245–8 DOI: 10.1038/362245a0.

Lai Z-W, Borsuk R, Shadakshari A, Yu J, Dawood M, Garcia R et al. Mechanistic target of rapamycin activation triggers IL-4 production and necrotic death of double-negative T cells in patients with systemic lupus erythematosus. J Immunol 2013; 191(5):2236–46 DOI: 10.4049/jimmunol.1301005.

Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 2005; 201(2):233–40 DOI: 10.1084/jem.20041257.

Latthe M, Terry L, MacDonald TT. High frequency of CD8 alpha alpha homodimer-bearing T cells in human fetal intestine. Eur J Immunol 1994; 24(7):1703–5 DOI: 10.1002/eji.1830240737.

Lazarevic V, Glimcher LH, Lord GM. T-bet: a bridge between innate and adaptive immunity. Nat Rev Immunol 2013; 13(11):777–89 DOI: 10.1038/nri3536.

Lu L, Barbi J, Pan F. The regulation of immune tolerance by FOXP3. Nat Rev Immunol 2017; 17(11):703–17 DOI: 10.1038/nri.2017.75.

Luckschander N, Pfammatter NS, Sidler D, Jakob S, Burgener IA, Moore PF et al. Phenotyping, functional characterization, and developmental changes in canine intestinal intraepithelial lymphocytes. Vet Res 2009; 40(6):58 DOI: 10.1051/vetres/2009042.

Luhtala M, Lassila O, Toivanen P, Vainio O. A novel peripheral CD4+CD8+ T cell population: Inheritance of CD8α expression on CD4+ T cells. Eur J Immunol 1997; 27(1):189–93 DOI: 10.1002/eji.1830270128.

Maina E, Devriendt B, Cox E. Food allergen-specific sublingual immunotherapy modulates peripheral T cell responses of dogs with adverse food reactions. Vet Immunol Immunopathol 2019; 212:38–42 DOI: 10.1016/j.vetimm.2019.05.003.

Marsella R, Benedetto A de. Atopic Dermatitis in Animals and People: An Update and Comparative Review. Vet Sci 2017; 4(3):37 DOI: 10.3390/vetsci4030037.

Martina MN, Noel S, Saxena A, Bandapalle S, Majithia R, Jie C et al. Double-Negative αβ T Cells Are Early Responders to AKI and Are Found in Human Kidney. J Am Soc Nephrol 2016; 27(4):1113–23 DOI: 10.1681/ASN.2014121214.

Masopust D, Soerens AG. Tissue-Resident T Cells and Other Resident Leukocytes. Annu Rev Immunol 2019; 37:521–46 DOI: 10.1146/annurev-immunol-042617-053214.

Mayassi T, Jabri B. Human intraepithelial lymphocytes. Mucosal Immunol 2018; 11(5):1281–9 DOI: 10.1038/s41385-018-0016-5.

McIver Z, Serio B, Dunbar A, O'Keefe CL, Powers J, Wlodarski M et al. Double-negative regulatory T cells induce allotolerance when expanded after allogeneic haematopoietic stem cell transplantation. Br J Haematol 2008; 141(2):170–8 DOI: 10.1111/j.1365-2141.2008.07021.x.

McKenzie BS, Kastelein RA, Cua DJ. Understanding the IL-23–IL-17 immune pathway. Trends Immunol 2006; 27(1):17–23 DOI: 10.1016/j.it.2005.10.003.

McNicol A-M, Bendle G, Holler A, Matjeka T, Dalton E, Rettig L et al. CD8α/α homodimers fail to function as co-receptor for a CD8-dependent TCR. Eur J Immunol 2007; 37(6):1634–41 DOI: 10.1002/eji.200636900.

Morrissey PJ, Charrier K, Horovitz DA, Fletcher FA, Watson JD. Analysis of the intra-epithelial lymphocyte compartment in SCID mice that received co-isogenic CD4+ T cells. Evidence that mature post-thymic CD4+ T cells can be induced to express CD8 alpha in vivo. J Immunol 1995; 154(6):2678–86.

Mosley RL, Styre D, Klein JR. CD4+ CD8+ murine intestinal intraepithelial lymphocytes. Int Immunol 1990; 2(4):361–5 DOI: 10.1093/intimm/2.4.361.

Mousset CM, Hobo W, Woestenenk R, Preijers F, Dolstra H, van der Waart AB. Comprehensive Phenotyping of T Cells Using Flow Cytometry. Cytometry 2019; 95(6):647–54 DOI: 10.1002/cyto.a.23724.

Mucida D, Husain MM, Muroi S, van Wijk F, Shinnakasu R, Naoe Y et al. Transcriptional reprogramming of mature CD4$^+$ helper T cells generates distinct MHC class II-restricted cytotoxic T lymphocytes. Nat Immunol 2013; 14(3):281–9 DOI: 10.1038/ni.2523.

Nakayama T, Hirahara K, Onodera A, Endo Y, Hosokawa H, Shinoda K et al. Th2 Cells in Health and Disease. Annu Rev Immunol 2017; 35:53–84 DOI: 10.1146/annurev-immunol-051116-052350.

Netea MG, Schlitzer A, Placek K, Joosten LAB, Schultze JL. Innate and Adaptive Immune Memory: an Evolutionary Continuum in the Host's Response to Pathogens. Cell Host Microbe 2019; 25(1):13–26 DOI: 10.1016/j.chom.2018.12.006.

Nielsen MM, Witherden DA, Havran WL. γδ T cells in homeostasis and host defence of epithelial barrier tissues. Nat Rev Immunol 2017; 17(12):733–45 DOI: 10.1038/nri.2017.101.

Nishida K, Kawashima A, Kanazawa T, Kidani Y, Yoshida T, Hirata M et al. Clinical importance of the expression of CD4+CD8+ T cells in renal cell carcinoma. Int Immunol 2020; 32(5):347–57 DOI: 10.1093/intimm/dxaa004.

Olivares-Villagómez D, van Kaer L. Intestinal Intraepithelial Lymphocytes: Sentinels of the Mucosal Barrier. Trends Immunol 2018; 39(4):264–75 DOI: 10.1016/j.it.2017.11.003.

Olivry T, Naydan DK, Moore PF. Characterization of the cutaneous inflammatory infiltrate in canine atopic dermatitis. Am J Dermatopathol 1997; 19(5):477–86 DOI: 10.1097/00000372-199710000-00008.

Otani I, Ohta K, Ishikawa A, Yamada T, Ishinazaka T, Ohtaki T et al. Flow Cytometric Analysis of Canine Umbilical Cord Blood Lymphocytes. J Vet Med Sci 2008; 70(3):285–7 DOI: 10.1292/jvms.70.285.

Overgaard NH, Cruz JL, Bridge JA, Nel HJ, Frazer IH, La Gruta NL et al. CD4+CD8β+ double-positive T cells in skin-draining lymph nodes respond to inflammatory signals from the skin. J Leukoc Biol 2017; 102(3):837–44 DOI: 10.1189/jlb.1AB0217-065R.

Overgaard NH, Jung J-W, Steptoe RJ, Wells JW. CD4+/CD8+ double-positive T cells: more than just a developmental stage? J Leukoc Biol 2015; 97(1):31–8 DOI: 10.1189/jlb.1RU0814-382.

Peeters D, Day MJ, Farnir F, Moore P, Clercx C. Distribution of leucocyte subsets in the canine respiratory tract. J Comp Pathol 2005; 132(4):261–72 DOI: 10.1016/j.jcpa.2004.10.003.

Pellicci DG, Koay H-F, Berzins SP. Thymic development of unconventional T cells: how NKT cells, MAIT cells and γδ T cells emerge. Nat Rev Immunol 2020 DOI: 10.1038/s41577-020-0345-y.

Perdomo-Celis F, Taborda NA, Rugeles MT. Follicular CD8+ T Cells: Origin, Function and Importance during HIV Infection. Front Immunol 2017; 8:1241 DOI: 10.3389/fimmu.2017.01241.

Pescovitz MD, Sakopoulos AG, Gaddy JA, Husmann RJ, Zuckermann FA. Porcine peripheral blood CD4+/CD8+ dual expressing T-cells. Vet Immunol Immunopathol 1994; 43(1-3):53–62 DOI: 10.1016/0165-2427(94)90120-1.

Pinheiro D, Singh Y, Grant CR, Appleton RC, Sacchini F, Walker KRL et al. Phenotypic and functional characterization of a CD4(+) CD25(high) FOXP3(high) regulatory T-cell population in the dog. Immunology 2011; 132(1):111–22 DOI: 10.1111/j.1365-2567.2010.03346.x.

Pistoia V, Tumino N, Vacca P, Veneziani I, Moretta A, Locatelli F et al. Human γδ T-Cells: From Surface Receptors to the Therapy of High-Risk Leukemias. Front Immunol 2018; 9:984 DOI: 10.3389/fimmu.2018.00984.

Priatel JJ, Utting O, Teh HS. TCR/self-antigen interactions drive double-negative T cell peripheral expansion and differentiation into suppressor cells. J Immunol 2001; 167(11):6188–94 DOI: 10.4049/jimmunol.167.11.6188.

Prinz I, Silva-Santos B, Pennington DJ. Functional development of γδ T cells. Eur J Immunol 2013; 43(8):1988–94 DOI: 10.1002/eji.201343759.

Qin G, Mao H, Zheng J, Sia SF, Liu Y, Chan P-L et al. Phosphoantigen-expanded human gammadelta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses. J Infect Dis 2009; 200(6):858–65 DOI: 10.1086/605413.

Ranera B, Antczak D, Miller D, Doroshenkova T, Ryan A, McIlwraith CW et al. Donor-derived equine mesenchymal stem cells suppress proliferation of mismatched lymphocytes. Equine Vet J 2016; 48(2):253–60 DOI: 10.1111/evj.12414.

Reboldi A, Cyster JG. Peyer's patches: organizing B-cell responses at the intestinal frontier. Immunol Rev 2016; 271(1):230–45 DOI: 10.1111/imr.12400.

Reinhardt C, Melms A. Normalization of elevated CD4-/CD8- (double-negative) T cells after thymectomy parallels clinical remission in myasthenia gravis associated with thymic hyperplasia but not thymoma. Ann Neurol 2000; 48(4):603–8.

Reubsaet L, Meerding J, Giezeman R, Kleer I de, Arets B, Prakken B et al. Der p 1-induced $CD4^+FOXP3^+GATA3^+$ T cells have suppressive properties and contribute to the polarization of the TH2-associated response. J Allergy Clin Immunol 2013; 132(6):1440–4 DOI: 10.1016/j.jaci.2013.06.028.

Rodríguez-Gómez IM, Talker SC, Käser T, Stadler M, Reiter L, Ladinig A et al. Expression of T-Bet, Eomesodermin, and GATA-3 Correlates With Distinct Phenotypes and Functional Properties in Porcine γδ T Cells. Front Immunol 2019; 10:396 DOI: 10.3389/fimmu.2019.00396.

Rothe K, Bismarck D, Büttner M, Alber G, Buttlar H von. Canine peripheral blood CD4 + CD8 + double-positive T cell subpopulations exhibit distinct T cell phenotypes and effector functions. Vet Immunol Immunopathol 2017; 185:48–56 DOI: 10.1016/j.vetimm.2017.01.005.

Rowshanravan B, Halliday N, Sansom DM. CTLA-4: a moving target in immunotherapy. Blood 2018; 131(1):58–67 DOI: 10.1182/blood-2017-06-741033.

Rudra D, deRoos P, Chaudhry A, Niec RE, Arvey A, Samstein RM et al. Transcription factor Foxp3 and its protein partners form a complex regulatory network. Nat Immunol 2012; 13(10):1010–9 DOI: 10.1038/ni.2402.

Rütgen BC, König R, Hammer SE, Groiss S, Saalmüller A, Schwendenwein I. Composition of lymphocyte subpopulations in normal canine lymph nodes. Vet Clin Pathol 2015; 44(1):58–69 DOI: 10.1111/vcp.12221.

Saalmüller A, Hirt W, Reddehase MJ. Porcine gamma/delta T lymphocyte subsets differing in their propensity to home to lymphoid tissue. Eur J Immunol 1990; 20(10):2343–6 DOI: 10.1002/eji.1830201026.

Saalmüller A, Reddehase MJ, Bühring HJ, Jonjić S, Koszinowski UH. Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. Eur J Immunol 1987; 17(9):1297–301 DOI: 10.1002/eji.1830170912.

Sadasivam M, Noel S, Lee SA, Gong J, Allaf ME, Pierorazio P et al. Activation and Proliferation of PD-1+ Kidney Double-Negative T Cells Is Dependent on Nonclassical MHC Proteins and IL-2. J Am Soc Nephrol 2019; 30(2):277–92 DOI: 10.1681/ASN.2018080815.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155(3):1151–64.

Sakaguchi S, Wing K, Miyara M. Regulatory T cells - a brief history and perspective. Eur J Immunol 2007; 37 Suppl 1:S116-23 DOI: 10.1002/eji.200737593.

Sato M, Kanemoto H, Kagawa Y, Kobayashi T, Goto-Koshino Y, Mochizuki H et al. Evaluation of the prognostic significance of BCL6 gene expression in canine high-grade B-cell lymphoma. Vet J 2012; 191(1):108–14 DOI: 10.1016/j.tvjl.2010.12.006.

Schütze N, Raue R, Büttner M, Alber G. Inactivated parapoxvirus ovis activates canine blood phagocytes and T lymphocytes. Vet Microbiol 2009; 137(3):260–7 DOI: 10.1016/j.vetmic.2009.01.035.

Seifert M, Küppers R. Human memory B cells. Leukemia 2016; 30(12):2283–92 DOI: 10.1038/leu.2016.226.

Silva-Santos B, Serre K, Norell H. γδ T cells in cancer. Nat Rev Immunol 2015; 15(11):683–91 DOI: 10.1038/nri3904.

Sinkora M, Stepanova K, Butler JE, Francis D, Santiago-Mateo K, Potockova H et al. Ileal Peyer's patches are not necessary for systemic B cell development and maintenance and do not contribute significantly to the overall B cell pool in swine. J Immunol 2011; 187(10):5150–61 DOI: 10.4049/jimmunol.1101879.

Sonea IM, Jergens AE, Sacco RE, Niyo Y, Merten E, Kauffman LK et al. Flow cytometric analysis of colonic and small intestinal mucosal lymphocytes obtained by endoscopic biopsy in the healthy dog. Vet Immunol Immunopathol 2000; 77(1-2):103–19 DOI: 10.1016/s0165-2427(00)00230-0.

Stebegg M, Kumar SD, Silva-Cayetano A, Fonseca VR, Linterman MA, Graca L. Regulation of the Germinal Center Response. Front Immunol 2018; 9:2469 DOI: 10.3389/fimmu.2018.02469.

Stein L, Bacmeister C, Ylaya K, Fetsch P, Wang Z, Hewitt SM et al. Immunophenotypic Characterization of Canine Splenic Follicular-Derived B-Cell Lymphoma. Vet Pathol 2019; 56(3):350–7 DOI: 10.1177/0300985818823668.

Sujino T, London M, van Hoytema Konijnenburg DP, Rendon T, Buch T, Silva HM et al. Tissue adaptation of regulatory and intraepithelial $CD4^+$ T cells controls gut inflammation. Science 2016; 352(6293):1581–6 DOI: 10.1126/science.aaf3892.

Summerfield A, Rziha H-J, Saalmüller A. Functional Characterization of Porcine CD4+CD8+Extrathymic T Lymphocytes. Cell Immunol 1996; 168(2):291–6 DOI: 10.1006/CIMM.1996.0078.

Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment. Cell 2000; 100(6):655–69 DOI: 10.1016/S0092-8674(00)80702-3.

Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating Th1 immune responses. Annu Rev Immunol 2003; 21:713–58 DOI: 10.1146/annurev.immunol.21.120601.140942.

Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science 2002; 295(5553):338–42 DOI: 10.1126/science.1065543.

Takimoto H, Nakamura T, Takeuchi M, Sumi Y, Tanaka T, Nomoto K et al. Age-associated increase in number of CD4+CD8+ intestinal intraepithelial lymphocytes in rats. Eur J Immunol 1992; 22(1):159–64 DOI: 10.1002/eji.1830220124.

Taniguchi M, Harada M, Dashtsoodol N, Kojo S. Discovery of NKT cells and development of NKT cell-targeted anti-tumor immunotherapy. Proc Jpn Acad Ser B, Phys Biol Sci 2015; 91(7):292–304 DOI: 10.2183/pjab.91.292.

Tian D, Yang L, Wang S, Zhu Y, Shi W, Zhang C et al. Double negative T cells mediate Lag3-dependent antigen-specific protection in allergic asthma. Nat Commun 2019; 10(1):4246 DOI: 10.1038/s41467-019-12243-0.

Toepp AJ, Petersen CA. The balancing act: Immunology of leishmaniosis. Res Vet Sci 2020; 130:19–25 DOI: 10.1016/j.rvsc.2020.02.004.

Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2002; 2(10):735–47 DOI: 10.1038/nri911.

Tsuji M, Komatsu N, Kawamoto S, Suzuki K, Kanagawa O, Honjo T et al. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. Science 2009; 323(5920):1488–92 DOI: 10.1126/science.1169152.

Ueyama A, Imura C, Fusamae Y, Tsujii K, Furue Y, Aoki M et al. Potential role of IL-17-producing CD4/CD8 double negative αβ T cells in psoriatic skin inflammation in a TPA-induced STAT3C transgenic mouse model. J Dermatol Sci 2017; 85(1):27–35 DOI: 10.1016/j.jdermsci.2016.10.007.

van Looringh Beeck FA, Leegwater PAJ, Herrmann T, Broere F, Rutten VPMG, Willemse T et al. Tandem repeats modify the structure of the canine CD1D gene. Anim Genet 2013; 44(3):352–5 DOI: 10.1111/age.12002.

Vantourout P, Hayday A. Six-of-the-best: unique contributions of γδ T cells to immunology. Nat Rev Immunol 2013; 13(2):88–100 DOI: 10.1038/nri3384.

Voelkl S, Gary R, Mackensen A. Characterization of the immunoregulatory function of human TCR-αβ+ CD4- CD8- double-negative T cells. Eur J Immunol 2011; 41(3):739–48 DOI: 10.1002/eji.201040982.

Wang X, Quinn PJ. Lipopolysaccharide: Biosynthetic pathway and structure modification. Prog Lipid Res 2010; 49(2):97–107 DOI: 10.1016/j.plipres.2009.06.002.

Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. Immunity 2011; 35(3):337–48 DOI: 10.1016/j.immuni.2011.08.012.

Waring P, Müllbacher A. Cell death induced by the Fas/Fas ligand pathway and its role in pathology. Immunol Cell Biol 1999; 77(4):312–7 DOI: 10.1046/j.1440-1711.1999.00837.x.

Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: An Effector CD4 T Cell Lineage with Regulatory T Cell Ties. Immunity 2006; 24(6):677–88 DOI: 10.1016/j.immuni.2006.06.002.

Wiarda JE, Trachsel JM, Bond ZF, Byrne KA, Gabler NK, Loving CL. Intraepithelial T Cells Diverge by Intestinal Location as Pigs Age. Front Immunol 2020; 11:1139 DOI: 10.3389/fimmu.2020.01139.

Wieczorek M, Abualrous ET, Sticht J, Álvaro-Benito M, Stolzenberg S, Noé F et al. Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation. Front Immunol 2017; 8:292 DOI: 10.3389/fimmu.2017.00292.

Wohlfert EA, Grainger JR, Bouladoux N, Konkel JE, Oldenhove G, Ribeiro CH et al. GATA3 controls Foxp3$^+$ regulatory T cell fate during inflammation in mice. J Clin Invest 2011; 121(11):4503–15 DOI: 10.1172/JCI57456.

Xiao X, Li K, Ma X, Liu B, He X, Yang S et al. Mucosal-Associated Invariant T Cells Expressing the TRAV1-TRAJ33 Chain Are Present in Pigs. Front Immunol 2019; 10:2070 DOI: 10.3389/fimmu.2019.02070.

Young KJ, DuTemple B, Phillips MJ, Zhang L. Inhibition of graft-versus-host disease by double-negative regulatory T cells. J Immunol 2003; 171(1):134–41 DOI: 10.4049/jimmunol.171.1.134.

Young KJ, Yang L, Phillips MJ, Zhang L. Donor-lymphocyte infusion induces transplantation tolerance by activating systemic and graft-infiltrating double-negative regulatory T cells. Blood 2002; 100(9):3408–14 DOI: 10.1182/blood-2002-01-0235.

Zamoyska R. The CD8 coreceptor revisited: One chain good, two chains better. Immunity 1994; 1(4):243–6 DOI: 10.1016/1074-7613(94)90075-2.

Zhang D, Yang W, Degauque N, Tian Y, Mikita A, Zheng XX. New differentiation pathway for double-negative regulatory T cells that regulates the magnitude of immune responses. Blood 2007; 109(9):4071–9 DOI: 10.1182/blood-2006-10-050625.

Zhang D, Zhang W, Ng TW, Wang Y, Liu Q, Gorantla V et al. Adoptive cell therapy using antigen-specific CD4$^-$CD8$^-$T regulatory cells to prevent autoimmune diabetes and promote islet allograft survival in NOD mice. Diabetologia 2011; 54(8):2082–92 DOI: 10.1007/s00125-011-2179-4.

Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J Biol Chem 1997; 272(34):21597–603 DOI: 10.1074/jbc.272.34.21597.

Zhang Z, Young K, Zhang L. CD3+CD4–CD8– αβ-TCR+ T cell as immune regulatory cell. J Mol Med 2001; 79(8):419–27 DOI: 10.1007/s001090100238.

Zhang ZX, Yang L, Young KJ, DuTemple B, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. Nat Med 2000; 6(7):782–9 DOI: 10.1038/77513.

Zheng W-p, Flavell RA. The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells. Cell 1997; 89(4):587–96 DOI: 10.1016/S0092-8674(00)80240-8.

Zhu J. T Helper Cell Differentiation, Heterogeneity, and Plasticity. Cold Spring Harb Perspect Biol 2018; 10(10) DOI: 10.1101/cshperspect.a030338.

Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q et al. Conditional deletion of Gata3 shows its essential function in T H 1-T H 2 responses. Nat Immunol 2004; 5(11):1157–65 DOI: 10.1038/ni1128.

Zuckermann FA. Extrathymic CD4/CD8 double positive T cells. Vet Immunol Immunopathol 1999; 72(1-2):55–66 DOI: 10.1016/S0165-2427(99)00118-X.

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